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Isolation and Biochemical Characterization of Acid- and Bile-Tolerant Strains of Lactobacillus Acidophilus and Bifidobacterium Bifidum

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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF ACID- AND BILE-TOLERANT STRAINS OF *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM BIFIDUM*

by

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ABSTRACT

Isolation and Biochemical Characterization of Acid- and Bile-Tolerant Strains of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

by

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Lactic acid bacteria have been reported to be used as a health adjunct in food for many years. However, these health benefits have not been proven, and how these bacteria pass through the digestion process and remain viable in the human intestinal tract is still not clear. The aim of this work was to isolate mutants from *Lactobacillus acidophilus* or *Bifidobacterium bifidum* that could tolerate the conditions of the digestion process (low pH and bile conduction) and to characterize these isolated mutants.

Acid- and bile-tolerant mutants of *L. acidophilus* were isolated from parental strains successfully using natural selection techniques. These mutants survived and grew at conditions of pH 3.5 with 0.2% mixed bile salts added. After the selection, phenotypic characterization was identified to further clarify desirable traits for use as probiotic adjuncts in foods. These phenotypic characteristics included protease, aminopeptidase, ß-galactosidase, and bile salt hydrolase activity. Based on different protease, aminopeptidase, and ß-galactosidase activity, selected acid- and bile-tolerant mutants contained different growth characteristics compared with their parents. All the isolates tested showed different bile salt hydrolase activity, and this activity was not strain and medium dependent.

Plasmid profiles and fatty acid analysis were conducted to provide more information of these acid/bile tolerant isolates and whether or not they were mutants from their parent strains rather than only adapted variants. Results showed the acid-/bile-tolerant isolates contained different plasmid profiles and cell wall fatty acids compared with their parents, which indicated these isolates were mutants. Protein expression by two-dimensional gel electrophoresis showed different protein expression patterns between acid- and bile-tolerant mutants and their parents, further suggesting these isolates were mutants. We observed the protein production in parent strains decreased as the pH decreased, and protein expression in mutants remained the same as pH decreased.

Two of the proposed health benefits of probiotic bacteria are anticholesterol activity and antimicrobial activity. These were evaluated using selected acid- and bile-tolerant mutants. Results showed no decrease of cholesterol in the test medium during bacterial growth. The observed antimicrobial activity was due to the presence of active cells, and this may relate to the acid production during cell growth and not to the production of antimicrobial substances.

We concluded that the acid-/bile-tolerant isolates were mutants, and they survived and grew better in harsh environments compared with their parent strains. These mutants may be useful as a food adjunct in the future, but further study is needed to establish their use and possible probiotic benefits.

(86 pages)

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LIST OF SYMBOLS, NOTATION, DEFINITIONS

Abbreviation key:

ASP = acid shock proteins

ATCC = American type culture collection

ATR = acid tolerant response

BCA = bicinchoninic acid

BSH = bile salt hydrolase

CFE = cell free extract

CHD = coronary heart disease

DNP = dinitrophenol

IPTG = Isopropyl β-D-thiogalactopyranoside

NDM = nonfat dry milk

OPA = *o*-phthaldialdehyde

PBS = phosphate buffer saline

PepN = aminopeptidase N

***p*-NA** = *p*-nitroanilide

***p*-NP** = *p*-nitrophenyl

TE = Tris-EDTA

INTRODUCTION

Lactic acid bacteria are termed probiotic and have been used as a health adjunct in food for many years. These bacteria, mainly lactobacilli and bifidobacteria, may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improving lactose utilization, and anticarcinogenic activity (13, 20, 21, 23, 25, 55, 77, 78). One interesting therapeutic function is anticholesterol activity because high plasma cholesterol is associated with high risk of heart attacks (26, 27, 44). Lin et al. (44), in an *in vitro* and *in vivo* study of lactobacilli on cholesterol, concluded that a reduction in serum cholesterol requires large numbers of viable bacteria in the intestinal tract.

Another important therapeutic function is antimicrobial activity (27). Perdigon et al. (61) conducted an experiment using mice and reported that feeding milk fermented with individual *L. casei* or *L. acidophilus* did not protect mice from *Salmonella* infection. However, feeding milk fermented with both of the strains protected the mice from salmonellosis, and all the animals survived. Although these functions have been studied in the laboratory, because of improper experimental design and strain variation, the claimed benefits are still questionable, and clinical studies are needed to firmly establish health benefits (26).

Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth. As such, probiotic bacteria should be resistant to the enzymes in the oral cavity (e.g., amylase, lysozyme) (21), as well as have the ability to resist the digestion process in the stomach and the intestinal tract. Since probiotic organisms do not reside for extended time periods in the mouth, the resistance to oral enzymes is of minimal importance. However, further digestive processes have longer residence time, hence the need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine.

Cellular stress begins in the stomach, which has an average residence time of 90 min (4) and pH as low as 1.5 (42). After the bacteria pass through the stomach, they enter the upper intestinal tract, which contains bile acids. However, the concentration of bile in the human gastrointestinal system is varied, and it may not be possible to predict at any given moment (42). The intestinal tract is an environment composed of bile acids (glycocholate is the major group of bile salts in human intestinal tract) and food with an unpredictable, slightly acid pH. After traveling through this harsh environment, the organism establishes a population by attaching to the epithelium (10). Therefore, based on this harsh environment (stomach acid and bile), strains selected for use as probiotic bacteria must possess the ability to tolerate, survive, and grow in these conditions before they can provide any health function.

In addition to providing health benefits, probiotic bacteria must have several characteristics during growth, such as fast growth in laboratory media and survival during freezing. Once a strain is consumed and survives digestion, it needs to have essential enzyme systems that allow gathered nutrients to persist and grow in the intestinal tract. These enzymes include ß-galactosidase, protease, and intracellular peptidase. ß-Galactosidase is the first enzyme needed and has been used to reduce lactose intolerance. This enzyme hydrolyzes lactose to release glucose and galactose. However, the activity of this enzyme may be altered during growth in harsh conditions. For example, in bile-containing media, 0.15% oxgall increases ß-galactosidase activity, but 0.3% oxgall inhibited the enzyme activity (56). The authors concluded the presence of bile increases the permeability of the cell wall, which allows more substrate into the cell, but oxgall inhibited enzyme activity at high concentration of bile. Therefore, high activity of ß-galactosidase is desirable for strains selected as a probiotic bacteria.

Protease is another enzyme that is needed for growth of lactic acid bacteria (11, 53, 64, 73). This enzyme degrades protein into smaller peptides and amino acids that are

required for growth. Additionally, intracellular aminopeptidases play a role in growth by further breaking down small peptides into essential free amino acids for nitrogen and energy sources (11, 53, 64, 73). For example, during cheese ripening, bacteria utilize amino acids to grow when the carbon source is depleted (64). Presumably, these enzyme systems would be important for growth in the intestinal tract as well. Therefore, screening for the phenotypic characteristics in delivery and persistence of strains selected as probiotic bacteria is necessary before they are added to food products.

Bile salt hydrolase (BSH) is another enzyme that has received more attention recently because it is involved in the growth of bacteria in an environment containing bile. It has also been linked to cholesterol metabolism, but both positive and negative observations exist (7, 8, 41, 76). This hydrolase exists in many bacteria and has been purified from *L. acidophilus* (63, 67, 68). Gastrointestinal bacteria can biotransform bile into a number of metabolites or deconjugate bile acids by BSH activity to survive such bile-containing conditions (29). Fernandes et al. (14) evaluated the effect of bile on the growth of bacteria in the upper intestinal tract and demonstrated that strains must possess BSH activity to survive the digestion process. These studies suggest that a probiotic adjunct must contain BSH activity to maintain a viable population in the intestinal tract.

Although use of lactobacilli as a dairy adjunct to provide therapeutic functions has had wide interest for many years, conditions in the human stomach during the digestion process are harsh for bacteria survival (i.e. low pH and bile content). Environmental-resistant mutants have not yet been developed for the use of reliable health adjuncts. Therefore, development of environment-resistant strains from lactobacilli and bifidobacteria is one of the objectives in this study.

To grow well in a harsh environment such as the gastrointestinal tract, bacteria must have certain functional proteins that are expressed to help cells against environmental stress like stomach acid (32, 37, 51). Investigation of new protein production is

necessary, and the results of this investigation can indicate the differences between parent strains and their derivative strains and the changes of metabolic pathway of those derivative strains.

Production of new proteins in bacteria is important to provide resistance to acid environments. This has been studied extensively in *Salmonella* and *Escherichia* (3, 15, 16, 17, 18, 32, 43, 45). Foster and Hall (19) examined the survival of *Salmonella typhimurium* during incubation in acid conditions (pH 7.7 to 3.3 for 175 min) and demonstrated that *S. typhimurium* dies rapidly below pH 3.8. However, if cells are exposed to a mild acid shock (pH 5.8) before incubation at low pH (pH 3.3), the culture survived better during short-term incubation (1.5 h) compared to the culture without a mild acid treatment. Foster calls this process "preshock" and refers to the difference in cellular response as the "acid tolerance response" (ATR). The ATR induces expression of a unique set of proteins that protect cells from acid damage and allow growth in a more severe acid environment (3.0-4.0). The ATR model includes two stages of cellular response: The first stage, pre-acid shock at pH 6.0, induces synthesis of a specific ATR homeostasis mechanism against a specific pH. In conjunction with the second stage, acid shock during which the cells shift directly from an alkaline environment (pH > 7.0) to an acid environment (pH < 4.5), cells become acid adapted (15, 16). The acid shock proteins (ASP), including at least 52 induced proteins, are expressed during preshock, and a set of constitutive proteins works together, which allow cells to become acid adapted.

Study of acid shock in lactic acid bacteria is limited. Kashket (37) stated lactic acid bacteria maintain a more alkaline condition in cytoplasm than in the medium during growth. This suggests that lactic acid bacteria tolerate a fairly wide range in internal proton concentration. McDonald et al. (51) studied the limiting lower internal and external cellular pH of *Leuconostoc* and *Lactobacillus* and mentioned that anaerobic microorganisms generally tolerated lower internal pH than other bacteria to decrease dependence on energy

consuming proton pumps. Shah and Jelen (69) studied the pH effect (pH 1.5-3.5) on the survival of lactic acid bacteria and found that survival of all four lactic cultures tested (*Lactobacillus delbruekii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Lactococcus lactis* ssp. *cremoris*) decreased during 2 h incubation in low pH conditions (pH 1.5-3.5), and the viable count was especially low at pH 1.5 and 2.5. Lankaputhra and Shah (42) confirmed the results of Shah and Jelen (69) and demonstrated that viable *L. acidophilus* counts decline rapidly at pH lower than 4.0, but no decrease was observed above pH 4.0. However, these studies showed results only when bacteria were exposed for a short time period (2-4 h) in acid conditions. The expression of acid-induced protein in *L. acidophilus* has not yet been investigated. We hypothesize acid-/bile-tolerant *L. acidophilus* and bifidobacteria isolates will produce new proteins that may change global regulatory functions that account for their metabolic pathway differences and increased resistance to acid and bile environments. To investigate this hypothesis, a two-dimensional polyacrylamide gel electrophoresis (PAGE) system was used to examine the protein expression in different pH (4.0, 5.2, and 6.8) environments in the cells treated with or without pre-acid shock.

OBJECTIVES

The hypothesis of this research is that isolation of acid-/bile-tolerant mutants from *Lactobacillus acidophilus* or *Bifidobacterium bifidum* is possible and that these isolates should survive better than the parent strains under a harsh environment.

The objectives of this work are:

1. To isolate acid- and bile-tolerant mutants from *Lactobacillus acidophilus* and *Bifidobacterium bifidum*;
2. To characterize isolated acid- and bile-tolerant mutants for enzyme activity, strain identification, and evaluation of possible health benefits;
3. To investigate protein expression shifts due to acid and bile tolerance.

LITERATURE REVIEW

Early interest in health benefits of lactic acid bacteria traces back to Eli Metchnikoff, who suggested people drink fermented milk containing lactobacilli to lengthen life (52). The bacteria described by Metchnikoff have recently been termed probiotic and are included in the human diet in an effort to improve general health. A probiotic bacteria should be capable of exerting beneficial effects on the host, be nonpathogenic and nontoxic, be present as viable cells in large numbers when consumed, be capable of surviving and metabolizing in the gut environment, and be stable and capable of remaining viable for long periods of time under storage and intestinal tract conditions (20). Therefore, selection of probiotic bacteria as dietary adjuncts is very important.

In 1979, Gilliland concluded that three species of bacteria have been most often suggested as dietary adjuncts, including *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* (23). Other commonly considered species of lactic acid bacteria used as a food adjunct are *Pediococcus acidilactici*, *Enterococcus faecium*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus*. These organisms possess the characteristics deemed important for probiotic bacteria, and they occur naturally in the human intestinal tract albeit in varying amounts. Fernandes et al. (13), in their review article, summarized the possible therapeutic roles of dietary lactobacilli and some possible benefits probiotic bacteria conferred in the human intestinal tract. This review compiles an extensive list of positive and negative studies and concludes lactobacilli provide therapeutic functions. Recent studies concerning the health benefits of probiotic bacteria have focused on improving nutritional value of food, decreasing serum cholesterol, inhibiting intestinal pathogens, increasing lactose utilization in the gut, and providing anticarcinogenic activity (27).

Many European-type fermented milk products containing live lactobacilli are widely available but have limited popularity in other areas of the world. Consumption of these products is recommended daily because lactobacilli do not persist in the gut beyond 5-7 d. Renner (65) compiled an exhaustive list of the physiological data and demonstrated that the scientific evidence connecting probiotic lactobacilli and improved health remains elusive and usually contradictory. Renner's examination finds many studies lack proper experimental design, do not have appropriate controls for valid comparisons and conclusions, and use different strains of probiotic lactic acid bacteria. Further, he concludes differences in the literature are primarily due to the wide variety of uncharacterized strains used combined with the lack of strain persistence in the intestinal tract. Renner concluded that valid studies that use complete experimental design with well defined strains are needed. To meet Renner's suggestions and to determine health benefits, probiotic bacteria must at least survive the digestion process (23, 62) and persist in the gut. Cell viability and ability to persist in the intestinal tract depend on the food carrier, ingestion of large numbers of viable cells, use of normal microbial inhabitants from the host's intestinal tract, acid tolerance, and bile tolerance (65). Therefore, an important trait of a probiotic culture must be acid tolerance, thereby allowing passage through the stomach.

Acid Tolerance

Passage through the stomach is a transient acid stress for cells and lasts approximately 90 min (4), suggesting strains must be able to withstand acid shock to survive and continue into the intestine. This could be linked to the observation that high cell numbers must be ingested to demonstrate any probiotic effect. Conway et al. (10) found survival of *L. acidophilus* exposed to gastric juice is strain dependent, indicating acid tolerance is an important factor of strain characteristic for probiotic lactobacilli to begin colonization. Lack of acid tolerance is also a characteristic that adds to the confusion

regarding the beneficial role of probiotic bacteria. Therefore, isolation of acid-tolerant lactobacilli and bifidobacteria should be the first step in selecting possible probiotic strains.

Since the first problem probiotic bacteria must overcome is the acid environment of the stomach, the stressful environment may induce some specific genes to produce specific proteins in response to these stressful conditions. This hypothesis leads to the assumption that new proteins are induced in the acid tolerant response in bacteria during acid conditions. An example of this concept is the acid adaptation of *Salmonella typhimurium*. *Salmonella typhimurium* grows in a wide pH range from 5 to 9 under different growth conditions based on triggered pH homeostasis mechanisms that maintain a relatively constant pH inside the cells (15, 16). However, *S. typhimurium* survives at pH $\leq$ 5 for 1.5 h. Foster describes this ability as an acid-tolerant response (ATR), which consists of two stages with the first being pre-acid shock. Acid-tolerant response is triggered at pH < 6.0 and is characterized by the synthesis of specific proteins, which leads to pH homeostasis. The second stage is post-acid shock, which is triggered below pH 4.5. The second stage is distinctly different from the pre-acid stress and is characterized by a second set of proteins being synthesized and is coupled with an inducible homeostasis system to enhance the cell's survivability. The post-acid shock proteins may minimize the damage of DNA or protein denaturation inside cells.

Leyer and Johnson (43) and Foster and Bearson (18) demonstrated that acid adaptation alters cell surface hydrophobicity, specific outer membrane protein induction, and cellular resistance to additional stress conditions during incubation of *S. typhimurium* in acid. Foster and Bearson (18) used dinitrophenol (DNP) lethal screening strategy and successfully isolated several acid-sensitive mutants and found some mutants (*atr*B and *atr*C) exhibit normal or nearly normal post-acid shock-induced acid tolerance but not pre-acid shock ATR stage. Other mutants (*atr*D, *atr*F, and *atr*G) are unable to induce acid tolerance by using either pre-acid or post-acid shock methods. They conclude that cells

have a limited capability for the synthesis of acid shock proteins (ASP). In other words, if the pH homeostasis system (pre-acid shock stage) is not induced to temporarily maintain an internal pH suitable for protein synthesis, cells will not produce new protective proteins under severe acid environments. These data suggest that investigation of acid-induced proteins is important in selecting lactobacilli or bifidobacteria for use as a probiotic.

Bile Tolerance

Bile tolerance is another important factor to allow bacteria to grow in the intestinal tract (65). After lactic acid bacteria pass through the stomach (pH as low as 1.5), they encounter the bile-laden intestinal tract where colonization may occur. If the cells possess bile resistance ability, they will have a greater chance to survive and attach to the intestinal epithelium for population establishment (10, 33).

Presence of bile acids affects the growth of bacteria in the intestinal tract (35, 65). Free bile acids are considered as toxic metabolites, which inhibit growth or kill many bacteria (21). Therefore, in addition to the ability to survive the shock of the acid environment in the stomach, probiotic bacteria must possess the ability to grow in the presence of bile salts or at least maintain cellular viability (24). Different species of *L. acidophilus* have varying bile-tolerant abilities in growing in a medium containing 0.3% oxgall (25). Moreover, Lankaputhra and Shah (42) showed that six strains of *L. acidophilus* and nine strains of *Bifidobacterium* decreased in cell numbers during the first 3 h of incubation in media containing 0.0%, 1.0%, and 1.5% bile at pH 4.5, but they suggested some lactobacilli and bifidobacteria tested may be considered as dietary adjuncts. Based on these studies, the conclusion is that bile tolerance is strain dependent and is an important characteristic in selecting lactobacilli or bifidobacteria for use as a health adjunct.

The ability to deconjugate bile acids may relate to a strain's bile tolerance. If bacteria possess specific enzymes to act on bile acids, bacteria will be able to transform

these bile acids to other metabolites and survive (22). One such enzyme is bile salt hydrolase (BSH). Early research on BSH can be traced to Norman and Grubb (57), who demonstrated *Streptococcus faecalis* and *Clostridium perfringens* possess this enzyme. Later, Gopal-Srivastava and Hylemon (29) purified and characterized BSH from *Clostridium perfringens*. Gilliland and Speck (22) found different intestinal lactobacilli can deconjugate different bile acids, but most lactobacilli deconjugated sodium glycocholate and taurocholate under low oxidation-reduction conditions. Recently, Lundeen and Savage (48) also found bile salt deconjugation is related to the presence of BSH in lactobacilli. They purified two BSH enzymes from *L. acidophilus* strain 100-100. In subsequent studies, Lundeen and Savage (49) found bile salt deconjugation is actually catalyzed by four different polymeric forms of BSH (A, B, C, and D), which they purified from *Lactobacillus* ssp. strain 100-100. Their work indicates that more than one BSH exists; BSH is strain dependent, and these enzymes may be expressed differently depending on the bile mixture in which the strains are grown.

The link between cell survival and BSH has not been firmly established. Much effort has been put forward in trying to correlate the relationship between bile tolerance and bile salt deconjugation, but no consensus has been reached. Walker and Gilliland (76) correlated the relationship between bile tolerance and bile salt deconjugation and concluded there is no significant correlation between these two factors. Therefore, more studies are needed in this area to establish the role of BSH activity and cell survival in the gut.

Proposed Probiotic Health Benefits

After probiotic bacteria establish their population in the human intestinal tract with large cell numbers, they may provide several health benefits, such as antimicrobial activity, anticholesterol activity, lactose hydrolysis, and anticarcinogenic activity (13, 20, 21, 23, 25, 55).

Metabolism of cholesterol. High cholesterol levels are generally associated with high risk of coronary heart disease (CHD), and the reduction of plasma cholesterol can lower the risk of CHD (47). Therefore, mechanisms to reduce plasma cholesterol have received considerable attention. Consumption of certain dairy products containing *Lactobacillus acidophilus* has potential to decrease serum cholesterol levels (22), but the mechanism of reduction has not been elucidated.

It is believed that there is a relationship among bile tolerance, bile salt deconjugation, and reduction of cholesterol since probiotic bacteria have been linked to an anticholesterol function (25). Bile tolerance is an important characteristic that enables probiotic bacteria to survive and provide action in the intestinal tract. Bile salt deconjugation functions in the solubilization of cholesterol (76). However, the results are controversial. Gilliland et al. (22) correlated BSH activity with cholesterol reduction and further investigated their strains of *Lactobacillus* isolated from fecal samples of pigs and reported the decrease of cholesterol concentration in media with cell growth (25). They (25) concluded that the organism metabolizes cholesterol during anaerobic incubation in the presence of oxgall. This study leads to the hypothesis that a relationship may exist between reduction in serum cholesterol and the presence of probiotic bacteria. However, the reduction mechanism is still not clear. Lin et al. (44) found a large variation of the strains studied in vivo and suggested that at least 10^8 lactobacilli/ml are required to substantially decrease cholesterol in media. In a similar study, Gilliland and Walker (28) did not find a direct relationship between bile tolerance and cholesterol assimilation, but they did find the culture assimilating cholesterol has better capability to grow in the presence of bile. Therefore, they recommended that more *L. acidophilus* strains should be screened since this trait is strain dependent. Recently, Walker and Gilliland (76) correlated bile tolerance, bile salt deconjugation, and assimilation of cholesterol in strains of *L. acidophilus*. They showed no significant relationship among these factors, but strains assimilating cholesterol

expressed bile tolerance and bile deconjugation activity, and they concluded the reduction of cholesterol is due to cholesterol uptake by cells. Klaver and van der Meer. (41) demonstrated the decrease in cholesterol by *L. acidophilus* from the growth media is due to bile salt deconjugation activity and not from catabolism of cholesterol by the organism. In later research, Buck and Gilliland (7) clarified previous work (76) and stated that there is no significant relationship between bile salt deconjugation and cholesterol assimilation. They suggested using isolates of *L. acidophilus* from human intestinal origin as a dairy adjunct. Further screening of these strains is needed. No studies have been published relating cholesterol metabolism with the four types of BSH that were purified by Lundeen and Savage (49) although current literature suggests that a relationship exists between cholesterol metabolism and BSH. These previous studies lead to the questions: Do probiotic bacteria, such as *L. acidophilus*, assimilate cholesterol, and is BSH part of the mechanism in changing serum cholesterol levels?

Antimicrobial activity. Antimicrobial activity is another therapeutic function that has received attention and is strain dependent. Gordon et al. (30) showed *L. acidophilus* has the ability to inhibit the growth of staphylococci in the human intestinal tract although the mechanism of inhibition is not clearly understood. The antagonistic action could be through the production of bacteriocins, which some strains of lactobacilli produce, or via lactic acid production (36). It has been reported that the major inhibitory compounds produced by strains of lactic acid bacteria include lactic acid, hydrogen peroxide, and antibiotic-like substances (5). Several antibiotic-like substances such as acidolin, acidophilin, and lactocidin produced by various strains of *L. acidophilus* have been reported (31, 70, 74).

Fernandes et al. (14) showed antimicrobial activity of *L. acidophilus* is affected by media composition and is strain dependent. Bhatia et al. (5) used *L. acidophilus* against *Campylobacter pylori* and observed a significant growth suppression of *C. pylori*. In this

study, they concluded this inhibition effect may be related to lactic acid (an extracellular secretory product) but not the pH of this secretory product. Khedekar et al. (39, 40) showed strains of *L. acidophilus* restrict the growth of *Staphylococcus aureus* and mastitic *Escherichia coli*, and this inhibition reaction is temperature dependent being better at 37°C than at 15°C. They also observed different inhibition effects with a steady increase of pH, indicating inhibition is pH independent. However, in a later study, Ibrahim and Bezkorovainy (34) demonstrated the inhibitory effects of bifidobacteria on *E. coli* is based on both the production of acetic and lactic acids and the low pH of the environment. Therefore, questions still remain and need to be studied in this area.

Most of the health benefits claimed for probiotic are not firmly verified because of experimental variation. Also, lactic acid bacteria have not been approved for any reliable therapeutic functions (50). Therefore, careful selection of *L. acidophilus* strains is necessary to ensure that desirable benefits are provided. The hypothesis of this study is to isolate acid-/bile-tolerant probiotic bacteria that have the chance to survive transit through the stomach and resist the antimicrobial effects of bile. To test this hypothesis, we isolated acid-/bile-tolerant strains from *L. acidophilus* or *B. bifidum*, characterized these isolates biochemically, and evaluated their cholesterol assimilation and antimicrobial capabilities.

MATERIALS AND METHODS

Bacteria

Strains of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were purchased from American Type Culture Collection (ATCC) (Table 1). *L. acidophilus* strains were grown anaerobically in MRS broth (main components are glucose, peptone, beef extract, and yeast extract) for 16 to 24 h at 37°C while *B. bifidum* strains were grown anaerobically in MRS broth containing 1% cysteine (9) for 16 to 24 h at 37°C. Stock cultures were prepared by growing the strains for 16 to 24 h and inoculating (17%) them into sterile 12% NDM containing 2% glycerol. Each vial contained 1.5 ml, was frozen immediately, and stored for further use at -70°C. Each experiment used a stock freezer vial for medium inoculation.

TABLE 1. Bacteria used in this study.

ATCC Number	Bacteria	Original isolation source
521	*L. acidophilus*	unknown
4796	*L. acidophilus*	unknown
4962	*L. acidophilus*	unknown
11975	*L. acidophilus*	unknown
4356	*L. acidophilus*	human
33200	*L. acidophilus*	human
43121	*L. acidophilus*	pig rectum
11863	*B. bifidum*	unknown
15696	*B. bifidum*	infant intestinal
35914	*B. bifidum*	human feces

Acid-Tolerant Isolates Selection

Each parent strain of *L. acidophilus* and *B. bifidum* was grown once in MRS broth from the stock freezer vial for 16 to 24 h at 37°C before use in an experiment. Cysteine (1%) was added to MRS broth for growth of *B. bifidum*. After this incubation, cells were harvested by centrifugation (4,300 x *g*, 10 min, 4°C), washed three times in phosphate buffer saline (pH 7.0), inoculated (1%) into MRS broth acidified with concentrated hydrochloric acid to pH 3.5 or unacidified MRS broth (pH 6.8), and incubated at 37°C in a Beckman DU-8 temperature-controlled spectrophotometer (Beckman, Fullerton, CA) for 90 min while monitoring the absorbance (650 nm) at 15-min intervals. Before and after incubation, total plate counts were done using MRS agar (pH 6.8) with the pour plate technique. Plates were incubated anaerobically at 37°C for 24 to 48 h. Strains that had little or no reduction in cell numbers after this treatment were considered to be candidates for selection of acid tolerant strains and were used to isolate 10 single colonies per strain from acidified MRS broth.

Each acid-tolerant candidate was further investigated for its ability to grow in acid conditions by streaking the organism onto acidified MRS agar (pH 3.5). Plates were incubated anaerobically at 37°C and observed for growth after 24 to 96 h. Ten individual colonies were selected and again grown on acidified MRS broth (pH 3.5) for 24 to 96 h at 37°C. Observed colonies were considered to be acid tolerant and were used to select acid and bile tolerant isolates.

Acid-tolerant isolates (10 single colonies) were preserved and prepared by growing the strains for 16-24 h under selection conditions and inoculating (17%) them into sterile 12% NDM containing 2% glycerol. Each vial, containing 1.5 ml, was frozen immediately and stored for further use at -70°C. Further studies with these isolates used individual freezer vials.

Bile-Tolerant Isolates Selection

Each acid-tolerant isolate was screened for bile tolerance using the direct plate assay described by Christiaens et al. (8). The MRS agar pH was adjusted to 4.0, 5.0, 6.0, and 7.0 and contained 0.3% of glycocholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, or oxgall, individually. Bile salt deconjugation was not tested below pH 4.0 because of bile salt precipitation. Each acid-tolerant isolate was struck at each pH level onto MRS agar containing individual bile salts and incubated anaerobically for 24 to 96 h at 37°C. Plates were observed for growth and bile precipitation at 24-h intervals. If growth occurred, 10 individual colonies for each strain were selected and inoculated into MRS broth containing the specific bile salt (0.3%) at the specific pH from which the isolate was selected. If growth were observed in this broth, the isolate was considered to be both acid and bile tolerant. Acid- and bile-tolerant isolates were preserved and prepared by growing the strains for 16 to 24 h under selection conditions and inoculating (17%) them into sterile 12% NDM containing 2% glycerol. Each vial contained 1.5 ml, was frozen immediately, and stored for further use at -70°C. Further studies with these isolates used individual freezer vials.

Acid-/Bile-Tolerant Isolate Verification

Frozen isolates were grown 16 to 24 h in their respective isolation broth to test strain stability after freezing. Cells were harvested by centrifugation (4,300 x *g*, 10 min, 4°C), washed three times with phosphate buffer saline (PBS, pH 7.0), and resuspended in the same volume of saline as the original culture. The first portion of the suspension was inoculated (1%) into acidified MRS broth only (pH 4.5) and into acidified MRS broth (pH 4.5) containing 0.2% of mixed bile salts (each bile salt at 0.05% concentration). Cultures were incubated in a temperature-controlled DU-65 spectrophotometer (Beckman, Fullerton,

CA) at 37°C for 600 min while monitoring the absorbance (650 nm) at 15-min intervals to determine the growth rate.

The second portion of the suspension was inoculated (1%) into acidified MRS broth (pH 4.5) while monitoring the absorbance (650 nm) at 15-min intervals. After 90 min of incubation at 37°C, 120 µl of sterile 1 N NaOH was added to the broth (giving a final pH of 6.7), and each bile salt was added to a final concentration of 0.2%. The absorbance (650 nm) was monitored for an additional 510 min at 15-min intervals at 37°C after the pH and bile salt adjustment.

Phenotypic Characterization of Acid-/Bile-Tolerant Strains

Bacteria. Strains that grew better at low pH and survived freezing tests were used to study the phenotypic characteristics (Table 2). Strains used in this part of the study were grown anaerobically in their respective isolation media for 16 to 24 h at 37°C. Each experiment used a stock freezer vial for medium inoculation to reduce culture variation.

TABLE 2. *L. acidophilus* used to study phenotypic characterization.

Strain	Isolation pH of MRS media	Comment
ATCC 43121	pH 6.8	Parent
LSC2	pH 3.5	Acid tolerant isolate
LSC2-1 GD4	pH 5.2	Acid/bile tolerant isolate
ATCC 33200	pH 6.8	Parent
LSC13	pH 3.5	Acid tolerant isolate
LSC13-1 GD4	pH 5.2	Acid/bile tolerant isolate

Cell free extract preparation. Cell-free extracts (CFE) from cultures grown in their respective isolation media were prepared as described by Dias and Weimer (12). Cultures were incubated for 14 h in their respective isolation media at 37°C and harvested from 10 ml of media by centrifugation at 7,000 x *g* for 10 min at 4°C. The cell pellet was collected and washed twice with 0.05 M sodium phosphate buffer (pH 7.2) before resuspension in 1 ml of 3 mM sodium phosphate buffer (pH 7.2) containing 200 U/ml mutanolysin, and 40,000 U/ml lysozyme. Cells were incubated in this lysis buffer for 1 h at 37°C. Glass beads (Sigma Chemicals, St. Louis, MO) were added, and the sample was vortexed at high speed for 2 min at room temperature to complete cell lysis. This mixture was considered to be the CFE and was used for intracellular enzyme assays.

β-glycosidase activity. Complex carbohydrate hydrolysis was determined in the CFE of each acid tolerant isolate and its parent using automated reflectance colorimetry (Omnispec® 4000 Bioactivity Monitor, Wescor, Inc., Logan, UT) and *p*-nitrophenyl saccharide (*p*-NP) derivatives (Sigma Chemical, St. Louis, MO). Stock solutions of chromogenic substrates (*p*-NP derivatives of β-D-glucopyranoside, α-D-glucopyranoside, β-D-galactopyranoside, α-D-galactopyranoside, β-D-cellobioside) were dissolved to 1.5 mM in sterile 0.05 M sodium phosphate buffer (pH 7.2) prior to each assay and frozen at -20°C. Prior to the enzyme assay, each stock substrate solution was thawed, equilibrated to the assay temperature, and mixed with CFE prior to testing. Each assay mixture contained 100 µl of 1.5 mM chromogenic substrate and 100 µl of CFE. Assays were carried out at 37°C in 96-well microtiter plates (Baxter Diagnostic, Inc., Deerfield, IL) with sterile tape coverings. Hydrolysis of each chromogenic substrate was measured at 10-min intervals for 10 h by monitoring the increase in yellowness (b*) in an Omnispec® 4000 Bioactivity Monitor (Wescor, Inc., Logan, UT). β-Galactosidase activity was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) as described by Miller (54). Three controls containing 100 µl of 1 mM chromogenic substrate in sodium

phosphate buffer (0.05 M, pH 7.2), 100 µl buffer, and 0.5 mM solution of *p*-NP in 0.05 M sodium phosphate buffer (pH 7.2) were also included. Means of duplicate assays are reported.

Protease activity. The activity of protease was determined using *o*-phthaldialdehyde (OPA) as described by Oberg et al. (58). Strains were grown anaerobically overnight at 37°C in their respective isolation broth, harvested by centrifugation (7,000 x *g* for 10 min at 4°C), washed three times with sterile saline (0.85% NaCl), and resuspended in sterile saline on OD_{590} of 0.4. The washed cell solution (200 µl) was inoculated into 10% NDM and incubated for 4.5 h at 37°C. Plate counts were conducted after the incubation and trichloroacetic acid was added to a 6% final concentration to stop the enzyme reaction and precipitate nonhydrolyzed protein. The assay mixture was filtered with #1 Whatman filter paper, and the supernatant was collected to react with OPA reagent. The supernatant and OPA reagent mixture (1:1 ratio) was incubated at room temperature for 5 min, and the absorbance at A_{340} was determined. Sterile NDM served as the control, and blank OPA reagent was used to zero the spectrophotometer. Means of duplicate assays are reported.

Aminopeptidase activity. Aminopeptidase activity was determined with chromogenic substrates using automated reflectance colorimetry as described by Dias and Weimer (12). Stock solutions (10 mM) of *p*-nitroanilide (*p*-NA) L-amino acid derivatives (Sigma Chemicals, St. Louis, MO) of arginine, leucine, lysine, alanine, valine, proline, methionine, glycine, and γ-glutamyl were dissolved in sterile 0.05 M sodium phosphate buffer (pH 7.2). *p*-Nitroanilide L-amino acid derivatives (Sigma Chemicals, St. Louis, MO) of tyrosine, phenylalanine, and S-benzyl-L-cysteine were dissolved in a minimum amount of 0.5 ml N, N-dimethyl formamide before addition to sterile 0.05 M sodium phosphate buffer (pH 7.2) to a final volume of 10 ml. Aliquots (1 ml) of 10 mM stock solutions were stored at -20°C. They were thawed and diluted immediately prior to use.

Each assay mixture contained 100 µl of 1 mM chromogenic substrate in 0.05 M sodium phosphate buffer (pH 7.2) and 100 µl of CFE. Assays were carried out at 37°C in 96-well microtiter plates (Baxter Diagnostic, McGaw Park, IL) with sterile tape coverings. Plates were preincubated at 37°C for 15 min before addition of enzymes. Hydrolysis of the chromogenic substrates was measured at 15-min intervals for 12 h by the increase in yellowness (b*) using the Omnispec® 4000 Bioactivity Monitor. Controls contained 100 µl of 1 mM chromogenic substrate, 100 µl sodium phosphate buffer (0.05 M, pH 7.2). Also, 0.5 mM solution of *p*-NA in 0.05 M sodium phosphate buffer (pH 7.2) served as a blank. Means of duplicate assays will be reported.

Protein determinations. Protein determinations were done according to manufacturer's instructions using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Enzyme activity calculation. Assays for aminopeptidase activity and β-glycosidase activity were done in three different Omnispec® 4000 Bioactivity Monitors, each with slightly different absolute color readings. Therefore, colorimetric values were standardized to eliminate error in the activity determination. Enzyme activities were determined calculating the slope of the linear portion of the curve, typically the first 1 h of the reaction, and adjusted for the amount of protein added in each assay (12).

Bile salt hydrolase activity. Acid-/bile-tolerant strains of *L. acidophilus* were grown anaerobically in their respective media for 16 to 24 h at 37°C (Table 3) for BSH activity. Stock cultures were prepared by growing the strains for 16-24 h and inoculating (17%) them into sterile 12% NDM containing 2% glycerol. Each vial contained 1.5 ml, was frozen immediately, and stored for further use at -70°C. Each experiment used a stock freezer vial for medium inoculation to reduce culture variation.

Whole cells were tested for BSH activity by following the release of glycine or tauroine using OPA. Reagents for the OPA assay were prepared as described by Oberg et

TABLE 3. Strains, isolation, and growth conditions used in BSH assay.

Strain	Growth & Isolation pH[a]	Growth & Isolation bile salt	Source
ATCC 43121	6.8	None	ATCC
LSC2-1 GD4	4.0	glycodeoxycholic	this study
LSC2-3 GD4	4.0	glycodeoxycholic	this study
LSC7-1 GD4	4.0	glycodeoxycholic	this study
ATCC 33200	6.8	None	ATCC
LSC13-1 GD4	4.0	glycodeoxycholic	this study
LSC14-1 TD5	5.0	taurodeoxycholic	this study
LSC14 GD4	4.0	glycodeoxycholic	this study

[a]Medium contains MRS at the indicated pH and bile salt.

al. (58). All strains were grown overnight at 37°C in acidified MRS broth (pH 5.2) containing a final concentration of 0.2% total of equal molar amounts of each bile salt (glycocholic acid, glycodeoxycholic acid, taurocholic acid, and taurodeoxycholic acid). Cells were inoculated (1%) into fresh MRS broth containing 0.1% of each bile salt and incubated at 37°C. The reason for using 0.1% of each individual bile salt was to maintain the selection pressure but not to stress the cells. Each culture was harvested by centrifugation (4,300 x *g*, 10 min, 4°C) after incubation of 0, 9, and 24 h for total plate count, BSH activity, and pH measurement. Harvested cells were washed twice with sterile saline followed by addition of 5 ml of each bile salt substrate (0.3% of each bile salt dissolved in 0.5 M phosphate buffer, pH 6.0) for BSH activity assay. The concentration of this bile salt substract (0.3%) was determined based on previous assay conditions (24, 28). This BSH assay mixture was incubated at 37°C for 30 min after which an equal volume of 20% TCA was added to each tube and incubated for another 30 min at 37°C. The supernatant was collected by centrifugation (4,300 x *g*, 10 min, 4°C) and mixed with 2

ml of the OPA reagent and incubated for 20 min at room temperature before measuring the absorbance at 340 nm. Assays were done in duplicate for all conditions.

Statistical analysis for BSH activity effects. Analysis of variance (ANOVA) analyzed the statistical significance of BSH activity. The model used was the multi-way factor ANOVA using single replication. The independent variables were strain, medium, substrate, and their interactions (strain-by-medium, strain-by-substrate, and medium-by-substrate). Error between two replications was very small, and we concluded that subsampling (sample taken from the same bottle each time) was used during the sample preparation. Therefore, replication was not counted as a variable during the statistic analysis. The three- way interaction term (strain-by-medium-by-substrate) was used as the error term.

Plasmid analysis. Small scale plasmid isolation was done as described by Anderson and McKay (2). After the isolation of plasmid DNA, the DNA pellet was dried in a vacuum desicator for at least 30 min, resuspended in 14 µl TE buffer (10 mM Tris, pH 8.0; and 1 mM EDTA), and electrophoresed (agarose, 0.6 to 0.7%) at 80 V for 2 h.

Fatty acid analysis. Selected strains were sent to Analytical Services, Inc. (Essex Junction, VT) to determine their cell-wall fatty acid content and identification.

Protein Expression of Acid-/Bile-Tolerant Mutants

Bacteria. Protein expression studies used strains that were acid-/bile-tolerant isolates obtained from previous procedures (Table 4). Selected strains were grown overnight in their respective isolation broth and transferred twice in MRS broth containing 0.2% mixed bile salts, pH 5.2, and grown overnight at 37°C before use. Each experiment used a vial from the frozen stock.

TABLE 4. Strains (*L. acidophilus*) used in two-dimensional electrophoresis gel system.

Strain	Condition[a]	Comment
ATCC 43121	6.8 5.2 4.0	Parent
LSC 2-1GD 4	6.8 5.2 4.0	Acid/bile tolerant isolates from ATCC 43121
ATCC 33200	6.8 5.2 4.0	Parent
LSC 13-1 GD 4	6.8 5.2 4.0	Acid/bile tolerant isolates from ATCC 33200

[a]pH at which the proteins were radiolabeled for one generation.

Two-dimensional electrophoresis gel. Stock cultures were inoculated (2%) into MRS broth (for ATCC parents) or MRS containing 0.2% mixed bile salts (glycocholic acid, glycodeoxycholic acid, taurocholic acid, and taurodeoxycholic acid) with pH 5.2 (for acid and bile tolerant mutants) and incubated at 37°C to an OD_{600} of 0.4 to 0.6. After incubation, cultures were harvested by centrifugation (4,300 x *g*, 4°C for 10 min), resuspended in 10 ml of fresh broth, and incubated for another 20 min at 37°C. Cells were collected by centrifugation (4,300 x *g*, 4°C for 10 min), resuspended in 1.5 ml of fresh MRS broth, and split into three microcentrifuge tubes containing 0.5 ml each. The microcentrifuge tubes were centrifuged at maximum speed for 1 min, and the cell pellets were resuspended in 0.7 ml of MRS of pH 6.8, 5.2, or 4.0 and incubated at 37°C for 5 min. After the 5-min incubation of the MRS broth at pH 6.8 and 5.2, 5 µl of [^{35}S]Met and [^{35}S]Cys were added to each microcentrifuge tube and incubated at 37°C for 1 generation (35 to 80 min). For labeling in MRS broth at pH 4.0, cells were pre-acid shocked at pH 5.2 and labeled for one generation (35 to 80 min) at pH 4.0 by adding 5 µl of [^{35}S]Met and

[^{35}S]Cys at 37°C. After one generation, 5 µl unlabeled Met and Cys were added to each tube to stop the labeling reaction. The radio isotope labeled cells were collected by centrifugation for 1 min at maximum speed and were washed once with 0.7 ml of fresh MRS broth at the respective pH, resuspended in 150 µl of TE buffer containing 250 mg/ml glass beads, vortexed for 8 min at maximum speed, centrifuged again (3 min at maximum, room temperature), and the supernatant was collected.

Two-dimensional gel electrophoresis (59) was described by the instruction manual of Protein® II xi Slab Cell (Bio-Rad, Richmond, CA). Supernatants were transferred to a new microfuge tube containing saturated urea, 30 µl of iso-electric focusing buffer (Bio-Rad, Richmond, CA), and 4 µl of Coomassie Blue R-250 (Bio-Rad, Richmond, CA). For first dimensional separation (iso-electric focusing), 40 µl of each sample mixture was added to the tubing gel. Isoelectric focusing was done in the first dimension tube gels at 400 V overnight and then increased to 800 V for 2 h on the second day. The second dimension used SDS-PAGE gels which were run at 30 milliamps/gel for the first 35 min, and then increased to 40 milliamps/gel for another 3.5 to 4 h. The resulting radioactive gel was put into a cassette with Kodax X-OMAT AR film (Eastman Kodak Co., Rochester N.Y. USA) and incubated in a dark room at room temperature for 5 d. The resulting protein patterns were analyzed after developing the autoradiograph.

Test of Potential Therapeutic Functions

Cholesterol metabolism. Pure cholesterol (Sigma Chemical, St. Louis, MO) was dissolved in 95% alcohol to a saturated concentration, filter sterilized, and added to MRS broth (pH 6.8) with 1:100 dilution for the test media. Before the assay, the concentration of cholesterol in the assay media (MRS broth containing alcohol with saturated cholesterol) was determined first using OPA as described by Rudel and Morris (66).

Stock cultures were grown overnight at 37°C in their respective media, inoculated (1.5%) into the MRS broth containing cholesterol, and this assay mixture was incubated at 37°C anaerobically for 12 h. Cells were removed from the test mixture by centrifugation (4,300 x *g*, 10 min, 4°C), and the remaining supernatant was adjusted to pH 6.5 to 6.8 and analyzed for cholesterol concentration using OPA (66). The absorbance (A_{550}) of the spent cholesterol/MRS medium was compared to a standard curve to determine cholesterol concentration before and after incubation.

Antimicrobial activity. Strains of *L. acidophilus* and the acid-/bile-tolerant isolates were grown overnight at 37°C in their respective isolation media. Six pathogenic strains (*Staphylococcus aureus*, *Salmonella arizonae*, *Escherichia coli*-0157:H7, *Shigella sonnei*, *Bacillus cereus*, and *Listeria monocytogenes*) were prepared in brain heart fusion broth and incubated overnight at 37°C.

Two assays were used to determine cellular inhibition by *L. acidophilus*. The first assay was the direct streak plate assay, which used one streak of the pathogenic organism placed on sheep blood agar followed by a streak of *L. acidophilus*, which was struck 90° across the pathogen. Plates were incubated at 37°C for 24 to 48 h. Growth of either *L. acidophilus* or the pathogenic organism was investigated after the incubation time. The second assay was a disc assay in which strains of *L. acidophilus* were grown overnight at 37°C in their respective broth; cells were centrifuged (4300 x *g*, 4°C for 10 min); the supernatant was collected, and the pH was adjusted to 6.5. Sterile paper discs (3.14 cm^2) were placed in this supernatant for 10 sec. Discs saturated with the supernatant were placed on sheep blood agar which contained a confluent lawn of the pathogenic strain. Plates were incubated at 37°C for 24 to 48 h. Clearing zones around the disc were measured after the incubation.

RESULTS AND DISCUSSION

Acid-Tolerant Isolate Selection

Food transition time through the human stomach is around 90 min (65). Therefore, each ATCC parent strain was tested for acid tolerance to pH 3.5 for 90 min. All strains tested were tolerant to pH 3.5 for 90 min at 37°C (Figure 1), but they exhibited a different growth response to this acid environment. Total plate counts (Table A1), done prior to and immediately following the 90 min incubation in the spectrophotometer, yielded the same information as the spectrophotometric growth curves verifying the data based on A_{650}. These data suggest acid tolerant variants exist in the parent population, and presumably, these variants can be isolated.

To isolate single colonies of acid tolerant strains, the cultures were inoculated into acidified MRS agar (pH 3.5) and incubated for 24 to 96 h. Despite resistance to pH 3.5 for 90 min each strain varied in its ability to produce colonies on MRS agar at pH 3.5. We observed *L. acidophilus* ATCC 11975 grew slightly in pH 3.5 broth during the 90 min incubation (Figure 1E), but produced no colonies on acidified MRS agar after 96 h, suggesting it had the ability to survive short-term acid stress, but not survive long-term exposure to acid environments. This observation was noted for many of the strains tested (Table 5).

If growth occurred on acidified MRS agar (pH 3.5), 10 single colonies were transferred to acidified MRS broth and incubated for 24 h at 37°C. If the isolate did not grow within 24 h of incubation in broth, it was considered to be too slow growing and was discarded from future work. We observed that *L. acidophilus* ATCC 43121, 33200, and 4962 produced colonies on acidified MRS agar and grew within 24 h in acidified MRS broth. Therefore, each of the 10 isolates (for a total of 30 isolates from three parent strains) was used in isolation of acid-/bile-tolerant cultures and for further characterization.

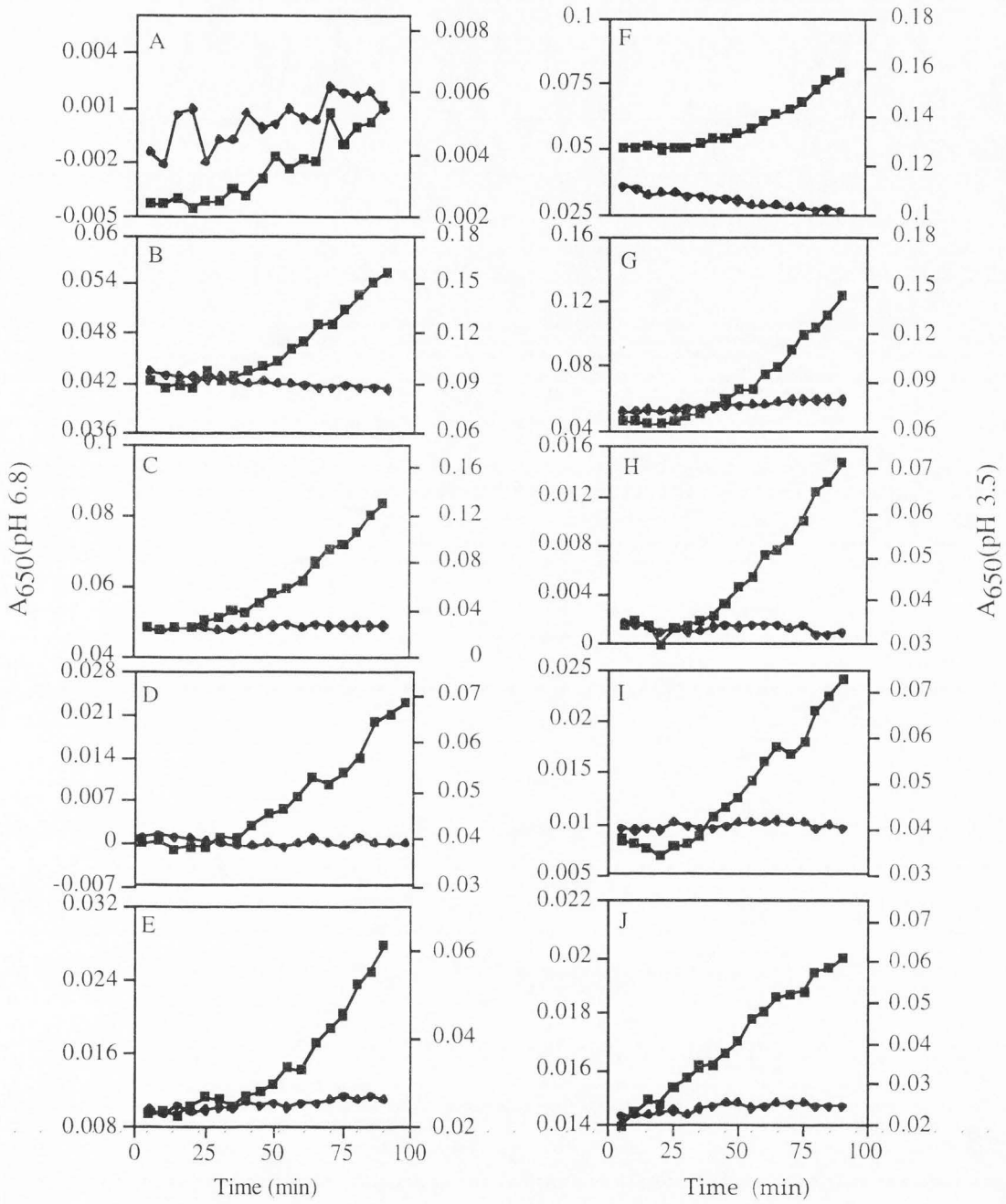

Figure 1. Spectrophotometric growth curves of strains: panels A-G are *L. acidophilus* ATCC 521, 4796, 4962, 4356, 11975, 33200, and 43121; panels H-J are *B. bifidum* ATCC 11863, 15696, and 35914. (—■— pH 6.8; —◆— pH 3.5)

TABLE 5. Parents yielding acid-tolerant isolates in this study.

Parent Strain (ATCC Number)	Time (h) for growth on acidified MRS agar[a]	Growth of isolates in acidified MRS broth[a]
L. acidophilus		
521	NG[b]	none
4796	NG	none
4962	10 colonies picked	10/10[c]
11975	NG	none
4356	NG	none
33200	10 colonies picked	10/10
43121	10 colonies picked	10/10
B. bifidum		
11863	10 colonies picked	none
15696	NG	none
35914	NG	none

[a]agar and broth pH was 3.5 with anaerobic incubation at 37°C.
[b]NG=no growth observed up to 96 h of anaerobic incubation at 37°C.
[c]10/10=10 out of 10 colonies picked grew in acidified MRS broth (pH 3.5) within 24 h.

Of the *B. bifidum* strains tested, *B. bifidum* ATCC 11863 grew slightly during the first 90 min incubation at pH 3.5 (Figure 1H) and was the only bifidobacteria to produce colonies on acidified MRS agar; however, no growth was observed in continuous incubation in acidified MRS broth (pH 3.5) within 24 h (Table 5). Since this was the only bifidobacteria to show promise for acid tolerance, it was used in experiments to isolate acid and bile tolerant strains (see the next section for data). These data suggested lactobacilli and bifidobacteria had the ability to survive and maintain viability for a short time in acid stress conditions, but this was a limited characteristic in *B. bifidum*.

Bile-Tolerant Isolate Selection

Each parent strain (*L. acidophilus* ATCC 43121, 33200, and 4962) and its acid-tolerant isolates were inoculated onto MRS agar containing individual bile salts at varying pH levels (pH 4.0 to 7.0) for bile tolerance isolate selection. Two distinct results were observed: growth indicated by a colony and bile salt deconjugation observed as a white precipitation surrounding the colony. Growth and deconjugation varied in each condition tested (Table 6). After 24 h of incubation, we observed that most of the strains tested grew at pH 5.0 to 7.0. However, with the addition of bile salts, pH 4.0 was the target pH in which we wanted the strain to survive. In pH 4.0 media, all strains tested were inhibited by glycocholic acid and oxgall. Three acid-tolerant isolates from ATCC 33200 grew in MRS containing taurocholic acid at pH 4.0. Only acid-tolerant isolate LSC 12 and ATCC 43121 grew in MRS containing taurodeoxycholic acid at pH 4.0. Most strains tested grew in MRS containing glycodeoxycholic acid at pH 4.0, except ATCC 33200 and LSC 15 (Table 6).

A precipitate-like substance was observed around the colonies on some of the plates. The production of this substance was interpreted as bile salt deconjugation during bacteria growth (8). We found parents ATCC 43121 and 33200 grew and deconjugated glycocholic acid at pH 5.0, but their acid-tolerant isolates grew but did not deconjugate bile under the same condition. Most strains tested deconjugated different bile salts at pH 5.0 to 7.0 with unpredictable patterns (Table 6). From these observations, it is suggested that more than one bile salt hydrolase may exist, and the deconjugation activity is strain, substrate (bile salt), and pH dependent. Based on these data, we hypothesized that isolation of bile-tolerant isolates from acid tolerant isolates was possible, the growth of bacteria was affected by different bile salt at varied pH range, and no predictable trend could be associated with growth or bile salt deconjugation. This indicated extensive strain characterization would be required to define the acid-/bile-tolerant isolates phenotype.

TABLE 6. Growth of parent and acid-tolerant strains on MRS agar at different pH and bile salts.

		Glycocholic acid[a]				Glycodeoxycholic acid				Taurocholic acid				Taurodeoxycholic acid				Oxgall			
		pH				pH				pH				pH				pH			
Strain	Isolate	4	5	6	7	4	5	6	7	4	5	6	7	4	5	6	7	4	5	6	7
ATCC 43121	Parent		G[b]D[c]	GD	GD	G	GD	GD	GD		GD	GD	GD	G	GD	GD	GD		GD	G	G
	LSC2		G	GD	GD	G		GD	GD		G	GD	GD		G	GD	GD		G	G	
	LSC7		G	GD	GD	G		GD	GD		G	GD	GD		G	GD	GD		G	G	GD
ATCC 33200	Parent		GD	GD	GD			GD	GD		GD	GD	GD		GD	GD	GD		GD	G	G
	LSC11		G	GD	GD	G		GD	GD		G	GD	GD		G	GD	GD		G	GD	GD
	LSC12		G	G	G	G		GD	GD			GD		G	G	GD	GD				GD
	LSC13		G	G	G	G		GD	GD	G	G	GD	GD		G	GD	GD		G	GD	GD
	LSC14		G	G	G	G		GD	GD		G	GD	GD		G	GD	GD			G	G
	LSC15		G	GD	G																
	LSC20		G	G	G	G			GD	GD	GD				GD				G		
	LSC20A		G	G	G	G		GD	GD	G	G	GD	GD		G	GD	GD				GD
ATCC 4962	Parent		GD	G	G	G	G		G		GD	GD	G		G	GD	GD			G	G
	LSC27					G															

[a]all media contained .3% of the respective bile salt
[b]G indicates growth
[c]D indicates bile salt deconjugation as measured by precipitation during growth (8)

The parent and acid-tolerant isolates of *B. bifidum* did not grow on any pH-adjusted MRS agar containing different bile salts. Therefore, use of *B. bifidum* was discontinued through the rest of the studies.

Our results demonstrated that with longer incubation times, bile-tolerant isolates grew, which showed the differences compared to Lankaputhra's work (42). An important difference in this study compared to previous reports is that we combined both acid and bile together during screening. A common observation among these studies, despite major design differences, is that acid and bile do have separate and joint effects on the growth of bacteria. Therefore, selection of acid-/bile-tolerant probiotic bacteria as a dairy adjunct needs to be part of a selection regime and should be done in combination.

Isolates that grew in the presence of bile salts at pH 4.0 or 5.0 were selected to verify the ability of these isolates to survive with long-term exposure to bile. This resulted in 42 presumptive acid-/bile-tolerant isolates. Acid and bile tolerance was verified in these isolates by inoculating them onto MRS agar at the isolation pH with mixed bile salts (glycocholic acid, glycodeoxycholic acid, taurocholic acid, and taurodeoxycholic acid). If the isolate grew within 24 h, it was considered to be a confirmed acid/bile tolerant isolate. Of the initial 42 presumptive acid-/bile-tolerant isolates, 18 isolates were confirmed to be acid and bile tolerant. These 18 acid-/bile-tolerant isolates were frozen (-70°C) and used for further studies.

Acid-/Bile-Tolerant Isolate Growth Characterization

Isolation conditions in this study defined acid-/bile-tolerant isolates by their ability to grow at low pH in the presence of bile salts within 24 h. Each isolate was subjected to: 1) freeze/thaw experiments to determine strain stability after the freezing effect, 2) growth at pH 6.8 vs pH 4.5, and 3) growth in environment containing both low pH and bile.

Influence of freezing on growth. Survival and rapid growth after freezing is an essential characteristic for probiotic bacteria because the mode of delivery is often in frozen dairy desserts. Additionally, it is critical to make stock cultures that are stored frozen. To investigate this ability, the 18 acid-/bile-tolerant isolates were frozen at -70°C for 2 d, thawed, and then regrown on MRS agar at pH 3.5 and 6.7. Many isolates readily grew in MRS agar at pH 6.7 after freezing; however, some of the isolates lost the ability to grow at pH 3.5 after freezing (Figure 2). Only isolates LSC2-1 GD4 and LSC13-1 GD4 grew equivalent to or better at pH 3.5 than pH 6.7. These results suggest that sensitivity to freezing is also an important characteristic in a probiotic selection program. Therefore, the

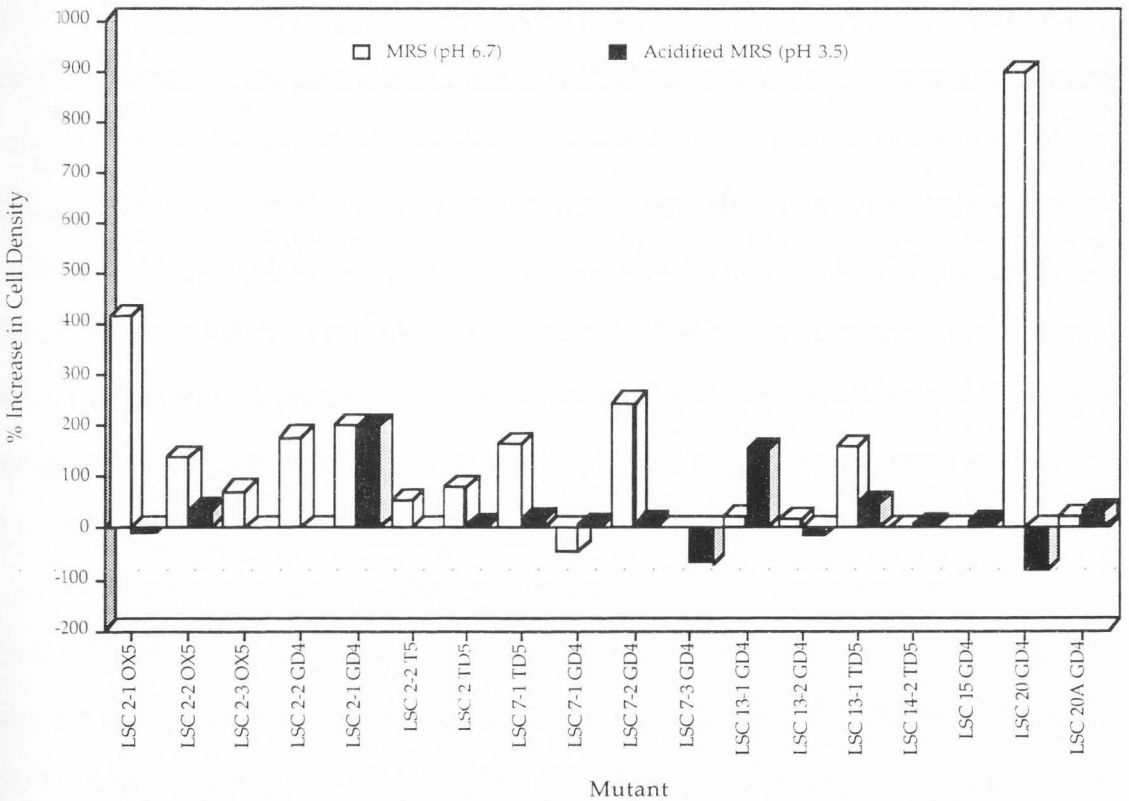

Figure 2. The effect of freezing on the ability of acid-/bile-tolerant mutants to grow in MRS (pH 6.7) and (pH 3.5). Percentage (%) increase in cell density was calculated by [(cell density after freezing effect)-(cell density before freezing effect)]/[cell density before freezing effect].

two isolates (LSC2-1 GD4 and LSC13-1 GD4) were used in further characterization studies (Figure 2).

Growth in neutral and acid conditions. In order to distinguish the characteristics of the acid-tolerant isolate from its parent, growth in both neutral and acidified conditions was investigated for changes in growth rate. Acid-tolerant isolate LSC13 was derived from ATCC 33200 by incubation on MRS agar at pH 3.5. In this verification step, we investigated only the influence of pH on the growth of LSC13 and its parent, ATCC 33200.

ATCC 33200 and acid-tolerant LSC13 were inoculated into MRS broth at pH 6.8 and 4.5 and monitored by absorbance (600 min) for growth (Figure 3). The parent grew well at pH 6.8 but grew only slightly at pH 4.5 and died after 7.5 h of incubation in both media. Conversely, the acid-tolerant mutant LSC13 grew as rapidly as its parent strain in nonselective conditions (pH 6.8) and did not rapidly die as the parent. When this acid-tolerant isolate was inoculated into acidified MRS broth (pH 4.5), it had a slightly longer lag time, but later grew as well as it did at pH 6.8, and did not die after 7.5 h of incubation. These data provided the first evidence to suggest that LSC13 was an acid-tolerant mutant rather than an acid-adapted variant since it grew equally well in selective (pH 4.5) and non-selective (pH 6.8) conditions. Additionally, LSC13 did not rapidly die upon entering stationary phase.

Growth in selective conditions was investigated further by mimicking the transit time, pH, and bile concentration in the human digestive tract to compare the growth of parent ATCC 33200, acid-tolerant isolate LSC13, and acid-/bile-tolerant isolate LSC13-1 GD4 (derived from LSC13). To determine the influence of low pH and bile on growth, the cultures were inoculated into acidified MRS broth (pH 3.5) for 90 min, followed by a pH increase to 6.8 with the addition of NaOH and 0.2% mixed bile salts. Growth was observed during 600 min of incubation at 37°C by spectrophotometry (Figure 4). After the

lag phase, *L. acidophilus* ATCC 33200 grew to a maximum cell density and died as quickly as in nonselective conditions (pH 6.8, no bile, Figure 3). However, acid-tolerant isolate LSC13 grew as fast as the parent, and remained in stationary phase over the incubation period, indicating this isolate may contain certain functional proteins that protect the cells, allowing better survival than its parent strain. Based on the results of the growth curve (Figure 4), lag time, and generation time (Table 7), the acid-tolerant isolate LSC13 may be a candidate for use as a probiotic adjunct. The acid-/bile-tolerant isolate LSC13-1 GD4 had a longer lag time and grew slower than LSC13 and its parent, but grew to a high cell density during the assay time. This suggests that this strain is a mutant but not an adapted variant. Also, LSC13-1 GD4 could be considered as a probiotic adjunct.

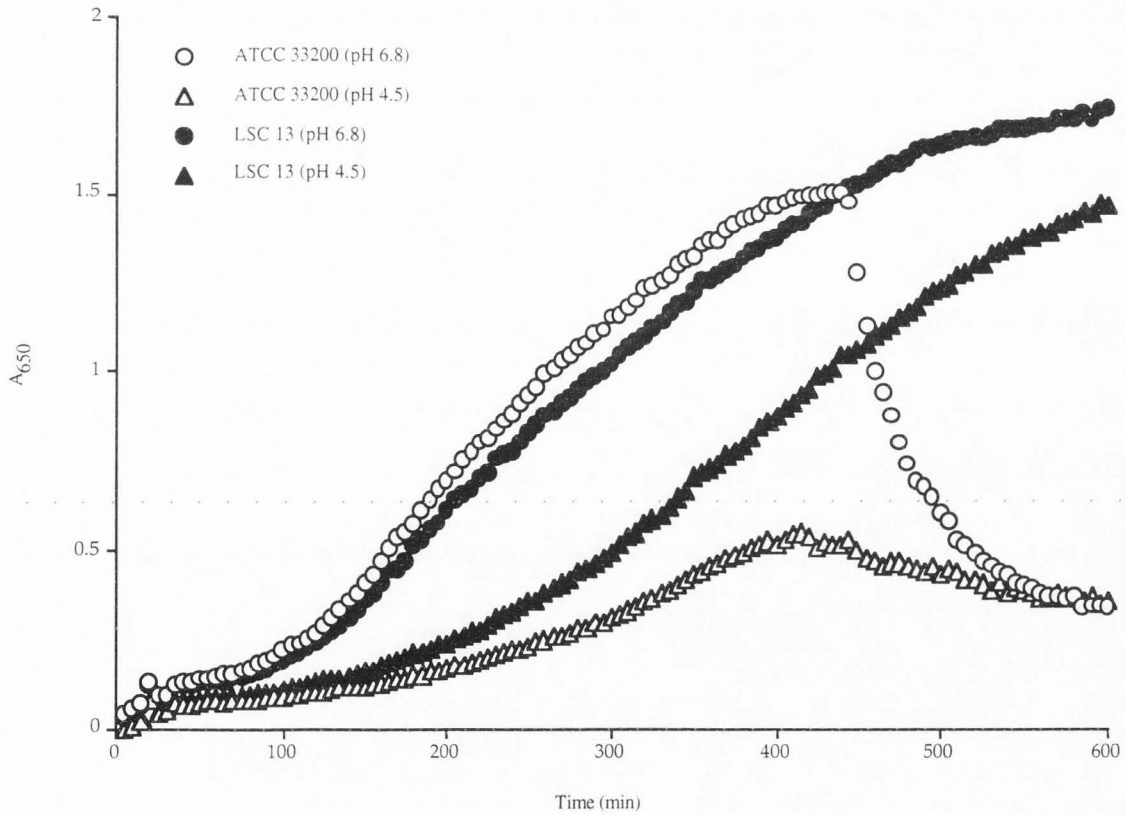

Figure 3. Growth of ATCC 33200 and acid-tolerant mutant LSC13 in MRS broth (pH 6.8) and acidified MRS broth (pH 4.5).

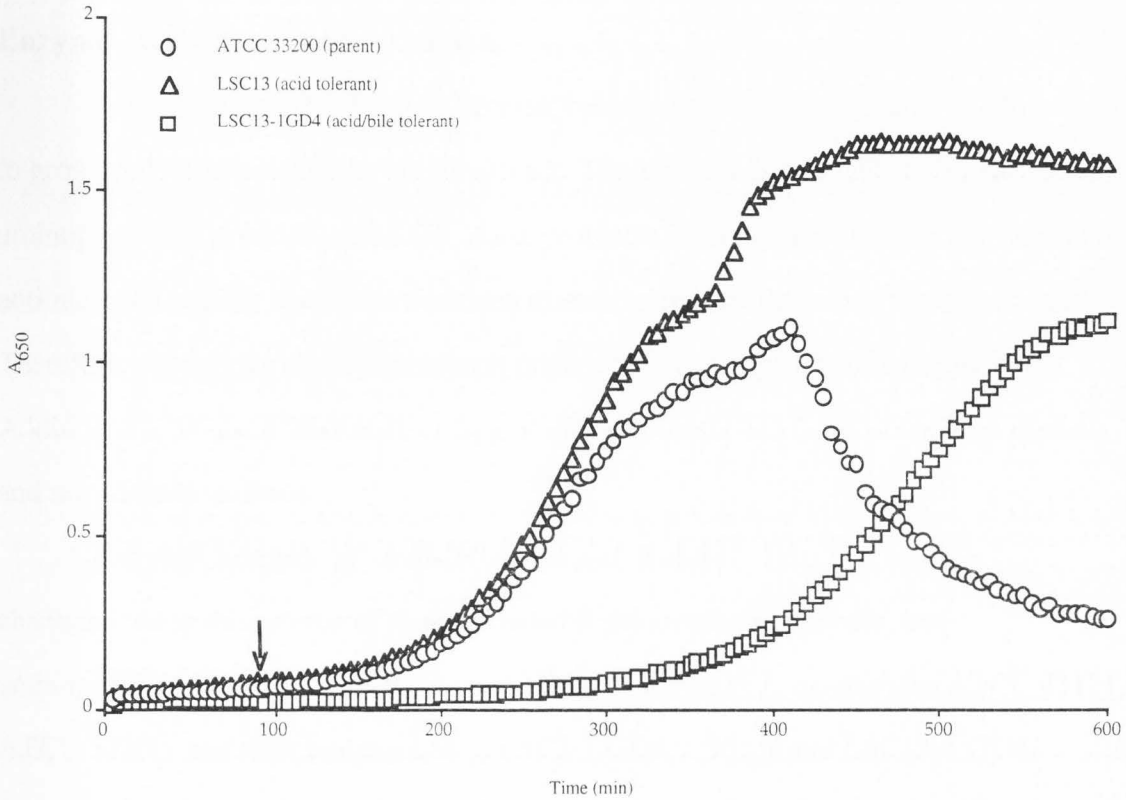

Figure 4. Growth of ATCC 33200, acid-tolerant mutant LSC13, and acid-/bile-tolerant mutant LSC13-1 GD4 in simulated gastric conditions. Arrow indicates time of NaOH/mixed bile salts addition to result in a 0.2% bile salt concentration at pH 6.8.

TABLE 7. Lag time and specific growth rate of *L. acidophilus* ATCC 33200 (parent), an acid-tolerant mutant (LSC13), and an acid-/bile-tolerant mutant (LSC13-1 GD4) grown in MRS broth containing 0.2% mixed bile salts and a pH increase to 6.8 after 90 min incubation at pH 3.5, simulating gastric conditions.

Strain	Lag Time (min)[a]	μ (h^{-1})
ATCC 33200	30	1.18
LSC13	35	1.16
LSC13-1GD4	125	0.82

[a]Calculated after addition of NaOH and mixed bile salts at 90 min.

Enzyme Activity Characterization

After surviving the digestion process, bacteria need enzyme systems to allow them to grow well and persist in the intestinal tract. These enzymes will include β-galactosidase, aminopeptidase, protease, and BSH. Also, probiotic functions such as anticholesterol and antimicrobial activity need to be evaluated to make sure bacteria deliver these functions. Therefore, phenotypic characterization is important to investigate before use in foods. Additionally, we used these tests to support our hypothesis that these isolates are mutants and not adapted variants.

Enzyme activity of acid-/bile-tolerant isolate. Enzyme activity characterized in this section of study included β-galactosidase, protease, and aminopeptidase activity. This work was limited to strains of *L. acidophilus* ATCC 43121, ATCC 33200, and their isolates LSC2, LSC2-1 GD4, LSC13, and LSC13-1 GD4, respectively, because these isolates had the best strain stability after freezing (Figure 2). In this section, we wanted to determine how different the selected isolates were as compared to their parents for phenotypic enzyme activity.

β-Galactosidase is an essential enzyme for lactobacilli to utilize complex carbohydrate sources such as lactose and has been shown to reduce the symptoms of lactose intolerance (65). We found that acid-/bile-tolerant isolate LSC13-1 GD4 had a lower β-galactosidase activity as compared to its parent ATCC 33200 and acid-tolerant isolate LSC13 (Table 8). In the ATCC 43121 strain series, all three strains showed low β-galactosidase activity as compared to ATCC 33200 series. After using IPTG to induce β-galactosidase activity, we observed β-galactosidase activity in strain ATCC 33200 and its isolates were inducible, suggesting the ATCC 33200 series are good probiotic candidates and can utilize lactose in the gut. However, this ability was not found in strain ATCC 43121 and its isolates.

Protease activity is also important because it is required by lactic acid bacteria for fast growth in dairy products (38, 73). The activity of ATCC 33200 and its isolates was about the same, but ATCC 43121 and its isolates had either very low or nondetectable activity as compared to the ATCC 33200 series (Table 8). These data suggest that the ATCC 43121 series will grow more slowly in milk than the ATCC 33200 series.

The ability to hydrolyze peptides for growth was investigated by monitoring the intracellular aminopeptidase activity (Table 8). The aminopeptidase test was done with individual substrates and CFE. Hydrolysis of each substrate varied, but no predictive pattern was found (Table A2). Therefore, the individual activities were summed and used to compare between the isolates (Table 8). Again, no predictable pattern was observed. Generally, the parent strains (ATCC 33200 and ATCC 43121) had a higher aminopeptidase activity as compared to their isolates. The general aminopeptidase N (PepN), defined based on the substrate specificity as Lys, Arg, and Leu (11), appeared to be impaired in acid/bile tolerant mutants.

The generation time of each series was tested in milk and MRS broth (pH 6.8). The parent ATCC 33200 grew significantly faster in MRS broth than in milk (Table 8). However, LSC13-1 GD4 grew significantly faster in milk than in MRS broth. This switch in growth rate between milk and MRS broth suggests LSC13-1 GD4 has a trait that is useful for dairy fermentation and may play a role in its ability to survive in dairy products for delivery as a probiotic agent. Based on the limited characteristics tested here, it seems plausible that high protease and an inducible β-galactosidase system may partially explain this switch, despite low total AP activity.

The ATCC 43121 series appears to be deficient in β-galactosidase and protease activity, which can account for their slow growth in milk, and suggests they are missing the plasmid containing these genes.

TABLE 8. Aminopeptidase activity, protease activity, β-galactosidase activity, and specific growth rate of selected parents, acid-tolerant mutants, and acid-/bile-tolerant mutants.

Enzyme Activity	Strain					
	ATCC 33200	LSC 13	LSC13-1 GD4	ATCC 43121	LSC 2	LSC2-1 GD4
Total AP Activity[a]	113	86	14	192	171	87
Protease Activity[b]	24	21	38	1	ND[c]	NA[d]
β-D-galactosidase (un-induced)[e]	22	20	1	1	1	7
β-D-galactosidase (IPTG induced)	77	69	29	1	1	9
Generation Time g(min)$^{-1}$						
MRS[f]	22±2^{X}	28±1^{X}	45±3^{X}	29±1^{X}	34±2^{X}	61±3^{X}
12% NFDM	38±4^{Y}	37±3^{Y}	25±2^{Y}	42±3^{Y}	42±4^{X}	44±5^{Y}

[a]unit=Δb*/CFU/ml
[b]unit=ΔA_{340}/CFU/ml
[c]ND=not determined
[d]NA=no detectable activity
[e]unit= Δb*/CFU/ml
[f]The same letter indicates no significant difference in the same column

BSH activity of acid-/bile-tolerant isolates. We investigated BSH activity because this enzyme may be involved in growth of bacteria in a bile-containing environment and may play a role in serum cholesterol reduction. BSH activity was determined using OPA method (58) and calculated based on the reading of A_{340} divided by cell numbers. All values exceeded the control after 9 h of incubation because BSH activity increased during the exponential growth phase and decreased in the stationary growth phase (Figures B1, B2).

Each parent and isolate tested had different BSH activities on each substrate tested (Table 9). However, the strains were not significantly different in their BSH activity ($P>0.05$). Media containing the deoxy form of bile acids significantly increased BSH activity ($P\leq0.05$).

The substrate specificity was significantly different ($P<0.05$), and it did not change when grown in different media ($P>0.05$). Interestingly, activity towards oxgall was significantly lower ($P<0.05$), suggesting this substrate does not accurately reflect BSH activity on other bile acids. The remaining bile acids were significantly different from each other ($P<0.05$). This observation suggests individual organisms have multiple enzymes, as observed by Lundeen and Savage (49). The implication of possessing different BSH enzymes is unknown. The benefit of having a specific BSH degradation enzyme is also unclear. Further work to define the role of BSH activity is required to assess the therapeutic application. One possible role of BSH is to metabolize bile in the intestinal tract and thereby force the equilibrium of cholesterol toward bile production. However, this is not proven.

Plasmid and cell wall fatty acid analysis. To further distinguish between mutant and adapted variants, plasmid and cell-wall fatty acid analyses were conducted since the main objective of this study was to isolate acid-/bile-tolerant *L. acidophilus* strains from a commercial parent with improved characteristics for use as of food adjuncts. Natural selection was used, and this technique is known to change many traits simultaneously and may produce adapted variants rather than a mutant (3, 18, 46). Therefore, distinguishing between a "mutant" and a "variant" is important because the variant may gain the desirable traits (acid and bile tolerance) temporarily, but a mutant will retain the traits when the selective pressure is removed. This was not investigated via DNA probes because the multiple genes that lead to the phenotype are not well known nor characterized. Therefore, a series phenotypic evidence was used to differentiate these states. These tests included

TABLE 9. ANOVA table of BSH activity against factor "strain," "media," and "substrate." Media used: cholic acid (G, T) and deoxycholic acid (D). Substrate used: oxgall, cholic acid (g, t), and deoxycholic acid (d).

SV	df	SS	MS	P
Strain	5	0.00024969	0.00004994	0.44
Media	3	0.00021502	0.00007167	0.25
GT	1	0.00000281	0.00000281	0.82
D	1	0.00021122	0.00021122	0.05
GT*D	1	0.00000099	0.00000099	0.89
Substrate	4	0.00141630	0.00035407	<0.00
oxgall vs rest	1	0.00118811	0.00118811	<0.00
among rest	3	0.00022819	0.00007606	0.23
gt	1	0.00058381	0.00058381	<0.00
d	1	0.00040449	0.00040449	0.01
gt*d	1	0.00036841	0.00036841	0.01
Strain*med	15	0.00075162	0.00005011	0.50
S*GT	5	0.00024962	0.00004992	0.44
S*D	5	0.00030992	0.00006198	0.32
S*GT*D	5	0.00019208	0.00003842	0.59
Strain*Sub	20	0.00129710	0.00006485	0.24
S*gt	5	0.00040658	0.00008132	0.18
S*d	5	0.00024184	0.00004837	0.46
S*gt*d	5	0.00048246	0.00009649	0.11
Med*Sub	12	0.00081496	0.00006791	0.23
GT*gt	1	0.00001024	0.00001024	0.66
GT*d	1	0.00000703	0.00000703	0.71
GT*gt*d	1	0.00000816	0.00000816	0.70

TABLE 9. Continued.

D*gt	1	0.00016602	0.00016602	0.08
D*d	1	0.00018054	0.00018054	0.07
D*gt*d	1	0.00022790	0.00022790	0.04
GT*D*gt	1	0.00001472	0.00001472	0.59
GT*D*d	1	0.00007286	0.00007286	0.24
GT*D*gt*d	1	0.00008993	0.00008993	0.19
Error	60	0.00309170	0.00005153	

growth in selective and nonselective conditions (Figure 3), enzyme activity characterization (Table 8), plasmid profiles (Figure 5), cell-wall fatty acid analysis (Figure 6), and protein expression analysis (described in the next section).

Initially, growth curve experiments that removed the selective pressure suggested the isolates were mutants because their growth characteristics were the same in selective and nonselective conditions (Figure 3). Further, aminopeptidase, protease, and β-galactosidase data showed these abilities were lost in some isolates, which suggested genes had been lost. Protease and β-galactosidase are known to be plasmid encoded (21) in these organisms and the plasmid is usually strain dependent with a size range from 57 to 78 Kb (75) in lactococci.

Plasmids are circular pieces of DNA that contain extra chromosomal genes important in the metabolism of protein (protease) and lactose (β-galactosidase or phospho-β-galactosidase) in lactic acid bacteria. Therefore, plasmid analysis is one of the useful tools to distinguish strains between mutation and adaptation. Analysis of selected isolates and parents showed different plasmid profiles (Figure 5). Parent strain ATCC 43121 contained two plasmids; one was 7045 base pair and the other was about 3990 base pair. Strain LSC2-1 GD4, the acid-/bile-tolerant isolate of ATCC 43121, lost both of the

plasmids, indicating this isolate possessed different genetic characteristic compared to its parent. This is consistent with the biochemical characterization for protease and lactose metabolism (Table 8) and explains why no protease and β-galactosidase activity were found. Another parent strain, ATCC 33200, also contained two plasmids, but these two plasmids were different compared to ATCC 43121. Its isolate, LSC13-1 GD4, lost both of the plasmids; however, this isolate still contains protease and inducible β-galactosidase activity, suggesting that these are chromosomally encoded in this isolate. Plasmid analysis suggested that the acid-/bile-tolerant isolates are mutants.

Cell-wall fatty acid analysis was done to further distinguish between parents and isolates. This technique is commonly used to identify bacteria since organisms have unique patterns of fatty acids in their cell wall, which allows identification if they are grown in the same conditions. We hypothesized that if the cell wall fatty acids are the same then the isolates are adaptation isolates and not mutants. Results showed two strains did not have the same fatty acid pattern (Table A3). Isolate LSC2-1 GD4 did not contain fatty acid 14:1 while its parent ATCC 43121 contained 0.9% of this fatty acid. The dendrogram comparison chart (Figure 6) of fatty acid analysis showed isolate LSC2-1 GD4 and its parent ATCC 43121 had a euclidian distance difference of less than 6 but more than 2, indicating these two cultures were not the same strain. The same results were observed for isolate LSC13-1 GD4 and its parent ATCC 33200 (Figure 6). These two strains also had different cell-wall fatty acid content (Table A4) and euclidian distance differences that were less than 6 but greater than 2, indicating they were not the same strains. These data further suggest that LSC2-1 GD4 and LSC13-1 GD4 are mutants and not acid-/bile-adapted variants.

Based on different growth characteristics in selective and nonselective conditions, different intracellular enzyme activity, different BSH activity presence, loss of plasmids, and different cell wall fatty acid composition, we confirmed our hypothesis and used

protein expression patterns in selective and nonselective conditions to further confirm our hypothesis.

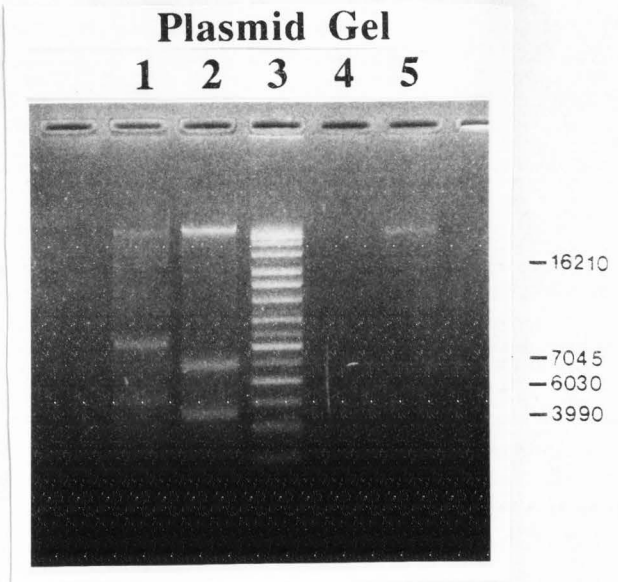

Figure 5. Plasmid analysis of strain ATCC 43121 (lane 1), ATCC 33200 (lane 2), supercoiled DNA ladder (lane 3), LSC2-1 GD4 (lane 4), and LSC13-1 GD4 (lane 5).

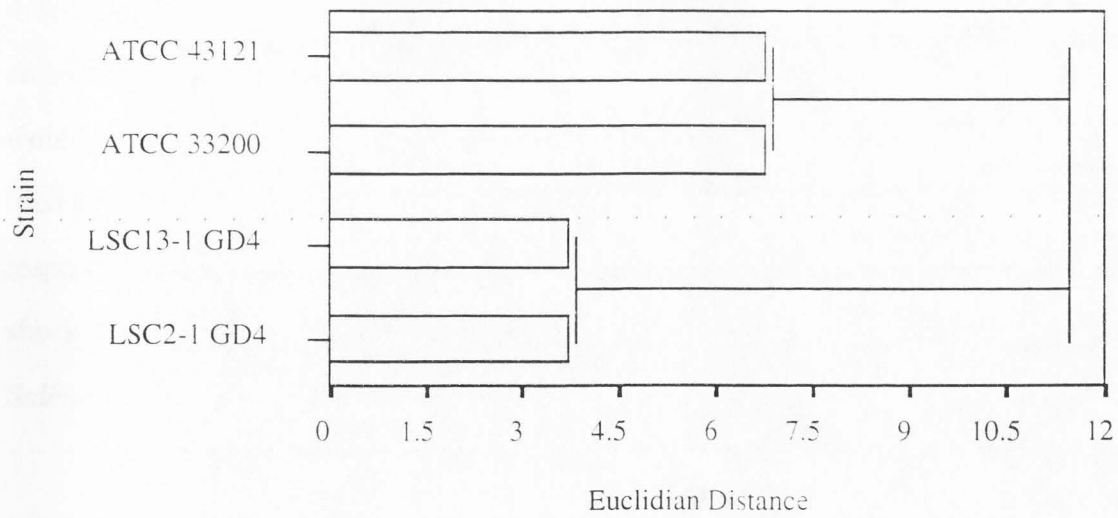

Figure 6. Dendrogram comparison chart of acid-/bile-tolerant isolates and their parents (fatty acid analysis). Less than or equal to 2.0= same strain; less than or equal to 6.0= same subspecies or biotype; less than or equal to 10.0= same species; less than or equal to 25.0= same genus.

Protein Expression of Acid-/Bile-Tolerant Mutants

Protein expression (measured by two dimensional SDS PAGE) is a technique widely used to investigate protein production during cell growth in transient stress conditions, especially acid shock (3, 18, 46). Since our results suggested the selected isolates were mutants of their parents and had enhanced ability to survive in acid and bile environment based on growth characteristics, enzyme activity, plasmid analysis, and fatty acid analysis, we hypothesized that the acid-/bile-tolerant isolates developed in this study express proteins differently and may account for some of the phenotypic differences. If this hypothesis is true, it suggests that a fundamental genetic change occurred during selection which is observable in the protein expression patterns of cells grown in non–selective conditions (radiolabel proteins at pH 6.8) and selective conditions (radiolabel proteins at pH 5.2 and acid shock at 5.2 with protein labeling at pH 4.0).

These experiments used multiple acid conditions to radiolabel the cellular proteins that were expressed in one generation of growth. As such, these experiments were designed to determine the influence of pH on protein expression in the parents and acid-/bile-tolerant isolates in an effort to explain the differences in growth and other phenotypic characteristics. The selected labeling conditions simulated the acid conditions that the cell would encounter during growth and digestion. Proteins labeled at pH 6.8 and 5.2 were used as the baseline measurements for no selective pressure and selective pressure, respectively. Labeling at pH 4.0 after an acid shock at pH 5.2 was used to simulate acid shock conditions to determine if this shock would increase protein expression as seen in *Salmonella* and *E. coli* (3, 18).

We observed differences in the protein expression pattern between all strains tested, suggesting this technique is useful in comparing strains irrespective of stress, and supporting previous characterization data that the isolates were mutants. At pH 5.2, protein

expression in the parents was severely restricted while expression in the acid-/bile-tolerant isolates had little or no difference as compared to pH 6.8, indicating that the acid-/bile-tolerant isolates have a greater complement of proteins being expressed in selective conditions than the parent. Presumably, the greater number of proteins, among them being stress proteins, allows the cell to have more metabolic functions, which then allows the cell to grow in acid. Preshocking the cells at pH 5.2 and then labeling the proteins at pH 4.0 further decreased protein expression in all strains tested, indicating that acid shock has no advantage for survival as observed in *Salmonella* and *E. coli* (3, 18). However, one universal conserved protein, located on the center of all gels, suggested this protein was required for strains to grow (spot 1 in Figures 7 to 10) (1). By comparing the information of blots and isoelectric position from *E. coli* homologs (71, 72), the apparent molecular weight (around 59 K), and the relative pI, it appears that spot 1 is GroEL (Table 10). This is a widely conserved heat shock protein that is important in protein folding (6) and known to occur in lactobacilli.

Table 10. Molecular weight (MW) of proteins detected by SDS two-dimensional PAGE.

Spot	Observed MW	Putative ID	Published MW	Comment
1	59,000	GroEL	62,883	expressed in all conditions
2	68,000	DnaK	69,121	found in ATCC 43121 and expressed in all pH conditions
3	72,000	ClpB	79,779	found in ATCC 43121 and expressed in all pH conditions
4	28,000	GrpE	21,668	decreased as pH decreased
5	47,000			decreased as the pH decreased in the parents, but present constitutively in the mutants
6	48,000			linked to acid tolerance

TABLE 10. Continued.

7	49,000	decreased as the pH decreased in the parents, but present constitutively in the mutants
8	51,000	linked to acid tolerance
9	27,000	linked to acid tolerance
10	27,000	linked to acid tolerance
11	67,000	linked to acid tolerance in the ATCC 33200 series
12	67,000	linked to acid tolerance in the ATCC 33200 series
13	50,000	unique to ATCC 33200
14	55,000	decreased as the pH decreased

When each strain grew at pH 6.8 and the proteins were labeled at the same pH, many proteins were expressed, and isolates showed a similar pattern compared to their parents (Figures 7a, 8a, 9a, 10a). In all strains tested at pH 6.8, spots 1 and 14 were expressed. However, spots 4 and 14 decreased in intensity as the pH decreased, suggesting that this protein was not needed, and the expression was repressed in acid conditions. Spot 2 was present only in ATCC 43121 and in its isolate LSC2-1 GD4, indicating strain differences, as did the biochemical characterization (Figures 7 and 8). Spot 3 was observed only in ATCC 43121 but not in its acid-/bile-tolerant isolate and ATCC 33200 series (Figures 7–10). Spot 3, putative identification of ClpB (Table 10), is a chaperone protein that stabilizes other stress proteins, renatures DnaK, acts as a protease that degrades denatured proteins in the cell, and is required for survival in heat shock (71). The impact of missing this protein in all strains tested except ATCC 43121 is unclear, but it can be concluded that it is not required for acid tolerance and growth.

In strains grown and labeled at pH 5.2, we observed the number of spots decreased in the parent strains, but not in the mutants, compared to pH 6.8 (Figure 7b, 8b, 9b, 10b). This protein pattern shift indicated the isolates were different from their parents, which supported previous characterization data, and the mutants were more acid tolerant than the parents. LSC2-1 GD4 and LSC13–1 GD4 had two new proteins (spots 9 and 10) expressed at this pH and pH 6.8, but the spots were not expressed when the cells were shocked at pH 5.2 and labeled at pH 4.0 (Figure 8b, 8c, 10b, 10c). These two proteins were expressed at a greater intensity at pH 5.2, suggesting that these proteins are linked to acid tolerance in the isolates, but their exact role in cell survival is unclear.

In addition to the conserved proteins, ATCC 43121 and its acid-/bile-tolerant isolate LSC2-1 GD4 expressed four proteins (spots 5–8) differently in response to pH. In the parent, this set of proteins was expressed at pH 6.8 and 5.2, but not acid shock at 5.2 with labeling at 4.0. Conversely, isolate LSC2-1 GD4 expressed these proteins at 5.2 and acid shock at 5.2 with labeling at 4.0. These observations suggest that these proteins were related to acid tolerance in this strain. These proteins, except spots 5 and 7, were not observed in the ATCC 33200 series. These proteins decreased in intensity with the acid

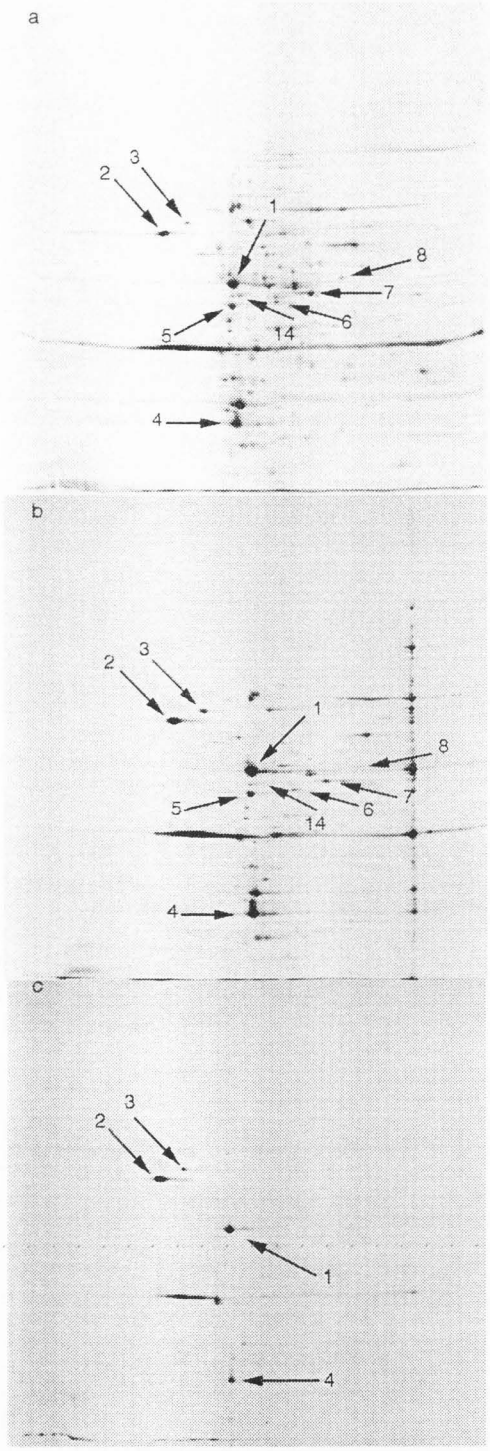

Figure 7. ATCC 43121 labeled at (a) pH 6.8, (b) pH 5.2, (c) pH 4.0 but preshock at pH 5.2. Arrows 1, 2, 3, 4, and 14 indicate the same spot.

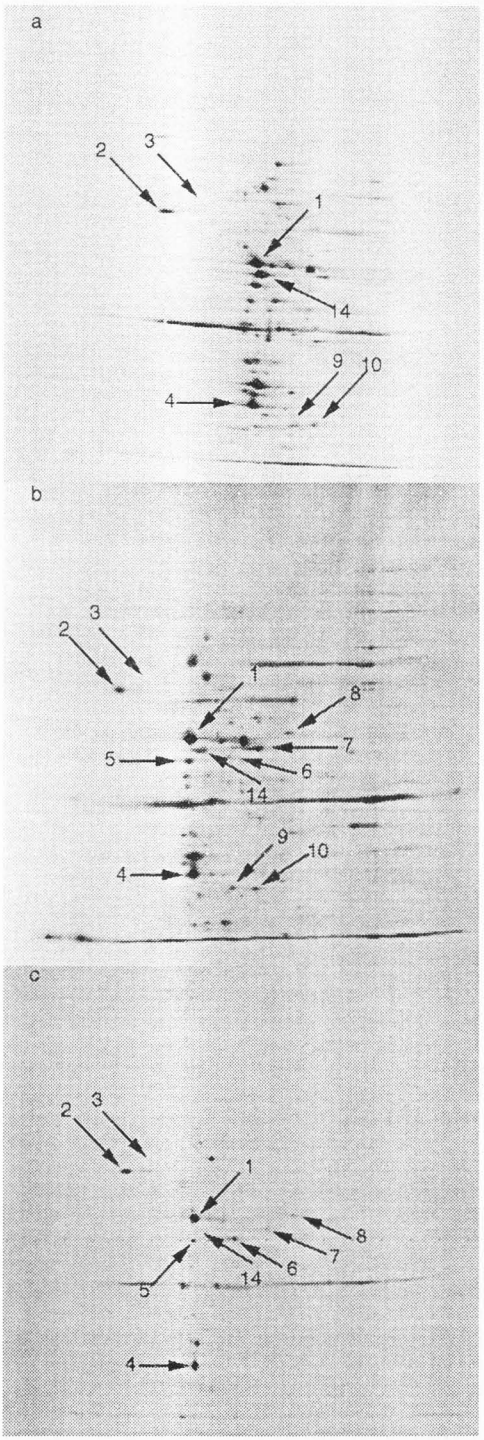

Figure 8. LSC2-1 GD4 labeled at (a) pH 6.8, (b) pH 5.2, (c) pH 4.0 but preshock at pH 5.2. Arrows 1, 2, 4, and 14 indicate the same spots; arrows 5-8 indicate spots shown at lower pH conditions; arrows 9-10 indicate spots shown at higher pH conditions.

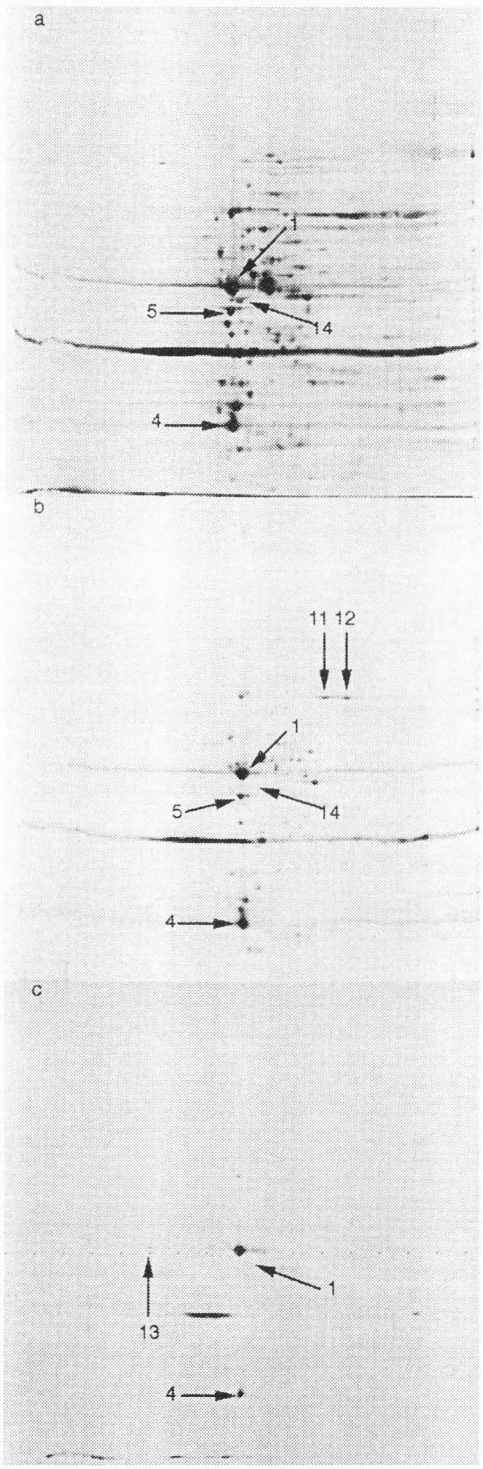

Figure 9. ATCC 33200 labeled at (a) pH 6.8, (b) pH 5.2, (c) pH 4.0 but preshock at pH 5.2. Arrows 1, 4, 14 indicate the same spots; arrows 11 and 12 indicate spots only shown at pH 5.2; arrow 13 indicates new spots shown at the lowest pH condition.

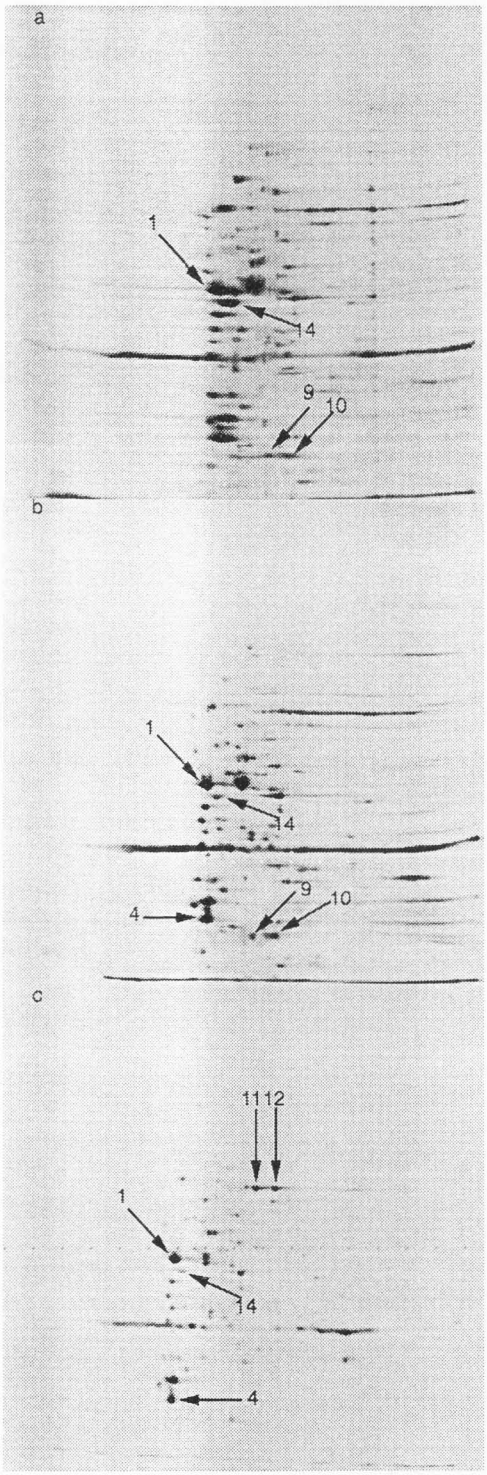

Figure 10. LSC13-1 GD4 labeled at (a) pH 6.8, (b) pH 5.2, (c) pH 4.0 but preshock at pH 5.2. Arrows 1 and 14 indicate the same spots; arrows 9 and 10 indicate spots shown at pH 6.8 and 5.2; arrows 11 and 12 indicate spots shown only at pH 4.0 with pH 5.2 preshock.

shock at pH 5.2, but are presumably linked to acid tolerance since they changed in expression with varying pH conditions.

In cells that were acid shocked at pH 5.2 and labeled at pH 4.0, we found the protein expression was further restricted compared to labeling pH 6.8 and 5.2 (Figure 7c, 8c, 9c, 10c). In the ATCC 43121 series, only the conserved proteins (spot 1, 2, 3, 4) existed in the parent, indicating acid shock did not induce a set of new proteins as has been observed in *Salmonella* and *E. coli* (3, 18). In the ATCC 33200 series, we observed a similar response to the other parent. In the acid-/bile-tolerant isolate, the protein expression pattern was also restricted, but many more proteins were observed as compared to the parent. Generally, the protein expression pattern of the parent at pH 6.8 was similar to the pattern of the respective acid-/bile-tolerant isolate at 5.2, suggesting that the isolates are more acid tolerant, which may account for their increased growth ability in acid conditions.

Based on the comparisons of all gel conditions, results indicated that there was an impact of acid. Parent strains did not have many proteins expressed after acid shock, but their mutants expressed many proteins under acid conditions. These results suggested acid-/bile-tolerant isolates were mutants with the ability to express more proteins in acid conditions, which led to improved survival in acid. Therefore, based on these results, combined with previous data, we concluded that the selection of acid-/bile-tolerant mutants was successful and that these mutants had growth advantages over their parents in a more stressed environment. However, further work is needed to define the role of specific proteins linked to acid tolerance identified in this work.

Test of Potential Therapeutic Functions

After confirming that these isolates were mutants and not adapted variants, we characterized them for their ability to metabolize cholesterol and inhibit pathogens. These functions were selected to represent two different capabilities: one to influence the

physiology of the host, and the other to influence the intestinal tract environment that has the potential to cause disease.

Cholesterol metabolism. In this experiment, the cholesterol concentration was determined before incubation of cells and used for reference values (Table A5). We observed no significant difference between strains to metabolize cholesterol during growth (Figure 11). Compared to previous studies, Gilliland et al. (25) demonstrated strains of *L. acidophilus* metabolized cholesterol during anaerobic growth and the presence of bile salts. However, Klaver and van der Meer (41) pointed out the coprecipitation of cholesterol during cell growth was due to bile salt deconjugation under acidified conditions. We added only cholesterol to the growth medium and did not add bile salts; therefore, the combined action of BSH and acidification would not be observed. Despite the difference in our experimental design to that of other reports, we did not observe a decrease in cholesterol during growth, supporting the conclusions of Klaver and van der Meer (41), suggesting that lactobacilli do not catabolize cholesterol.

Antimicrobial activity. This attribute was measured by using whole cells in the direct streak technique and with sterile culture supernatant in a disc assay. This approach determined if the physical presence of cells or a soluble component was needed to inhibit pathogens. Using the direct streak plate method, all strains except ATCC 33200 showed no inhibition against *Staphylococcus aureus*, *Salmonella arizonae*, and *Escherichia coli*. However, all strains tested showed positive inhibition to *Shigella sonnei* and partial inhibition to *Bacillus cereus* (Table 11). Only parent ATCC 33200 and isolate LSC13-1 GD4 were found to allow growth of *Listeria monocytogenes*.

In the disc assay (data not shown), the supernatant collected after cell growth was adjusted to pH 6.5 to eliminate the pH factor. Low pH could inhibit the growth of a pathogenic strain. No isolates inhibited any of the pathogens after incubation of 24 to 48 h

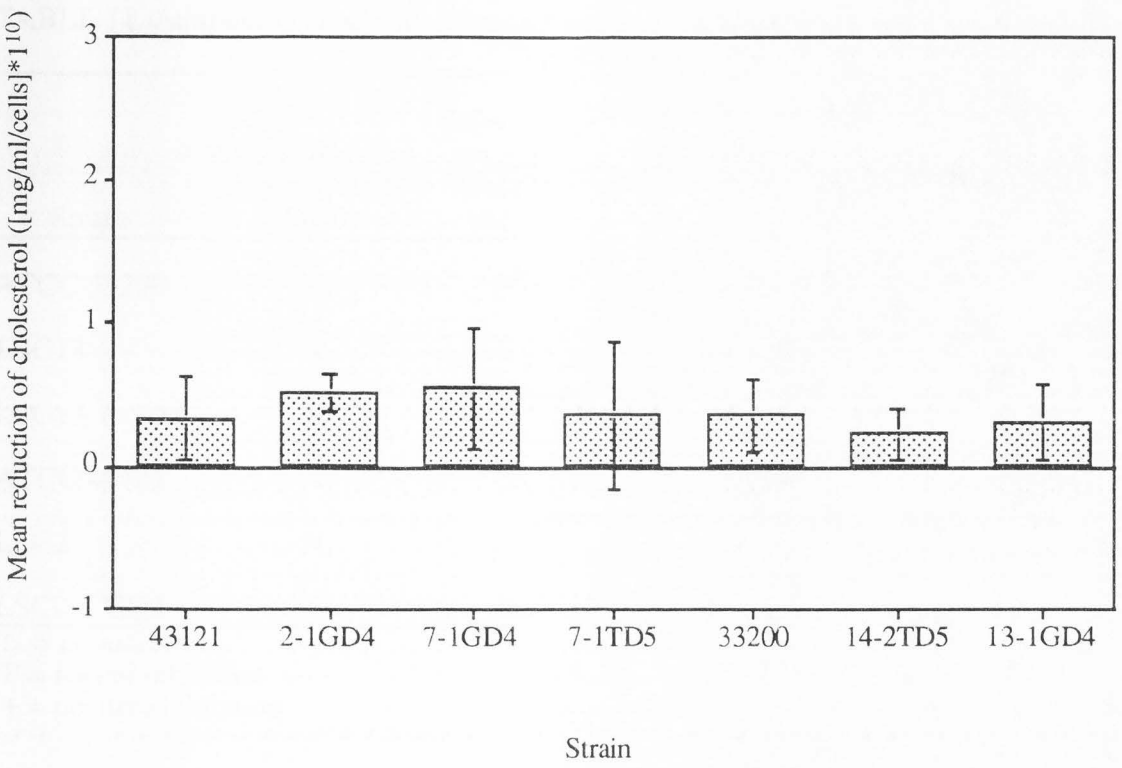

Figure 11. Reduction of cholesterol levels after incubate cells in cholesterol containing media. All values shown were adjusted by the proper control (see Table A5).

at 37°C. This observation suggests the supernatant produced by each strain tested had no antimicrobial substances produced during bacteria growth. Therefore, inhibition of pathogens required the presence of active cells that may compete for nutrients or produce lactic acid, which inhibits the pathogens.

TABLE 11. Antimicrobial activity using the direct streak assay.

Strain	*Staphylococcus aureus* (ATCC 12600)	*Salmonella arizonae* (ATCC 13324)	*Escherichia coli* (0157:H7)	*Shigella sonnei* (ATCC 25931)	*Bacillus cereus*	*Listeria monocy-togenes* (FDA 35152)
ATCC 33200	N[a]	P[b]	N	+[c]	P	N
LSC13	N	N	N	+	P	+
LSC13-1 GD4	N	N	N	+	P	N
ATCC 43121	N	N	N	+	P	+
LSC2	N	N	N	+	P	+
LSC2-1 GD4	N	N	N	+	P	+

[a]N = no inhibition
[b]P = partial inhibition
[c]+ = positive inhibition

CONCLUSIONS

Literature reports that *Lactobacillus acidophilus* and *Bifidobacterium bifidum* provide health benefits, such as a reduction of cholesterol levels and an antimicrobial activity, after people consume food products containing these probiotic bacteria. However, the results vary because of the lack of proper experimental design, strain variation, and lack of strain characterization (65). Therefore, the aim of this work was to generate acid-/bile-tolerant strains of *L. acidophilus* and *B. bifidum* that could survive the pH and bile content of the digestive tract and then to characterize some of their phenotypic characterization.

Using natural selection, we successfully isolated acid- and acid-/bile-tolerant mutants from *L. acidophilus*, but not from *B. bifidum*. These isolates were capable of rapid growth in MRS at pH 3.5 containing 0.2% mixed bile salts. Initial screening for acid resistance for 90 min did not accurately predict acid tolerance. Extended incubation in selective conditions was used to successfully isolate acid- and acid-/bile-tolerant colonies. Isolates were verified to be mutants rather than adapted variants based on growth in selective and non-selective conditions, freezing survival, biochemical characterization, plasmid profiles, cell-wall fatty acid analysis, and protein expression experiments.

Parsell and Lindquist (60) demonstrated the stress response (such as heat and acid) is highly conserved among different organisms and aids in their response to stress conditions in a similar way. Investigation of protein expression further confirmed the isolates to be mutants. Acid-/bile-tolerant mutants had a greater complement and differential expression of proteins in acid conditions, which could explain the increased survival observed in the mutants. Expression of a number of proteins was associated with growth in acid conditions, but their exact role in cell survival is unclear, and further work is needed to define how they aid the cell to grow in acid. After isolation and mutant verification, isolates LSC2-1 GD4 and LSC13-1 GD4 were observed to have the best growing ability under an acid and bile environment.

The selected acid-/bile-tolerant mutants and their parents did not reduce or metabolize cholesterol during growth. However, based on the results from previous studies (25, 41), there may be a relationship between presence of bile salts, BSH activity, acid production, and cholesterol precipitation. Mutants tested for antimicrobial activity showed inhibition of certain pathogenic strains, and we concluded this inhibition action was due to the whole cells. Therefore, use of these strains as a probiotic adjunct may provide protection from foodborne pathogens, but not reduce cholesterol.

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APPENDICES

Appendix A. Tables

TABLE A1. Plate count (Log_{10}) of *L. acidophilus* and *B. bifidum* before (0 min) and after (90 min) of incubation at pH 3.5.

	Control (pH 6.8)		Treatment (pH 3.5)	
Strain	0 min	90 min	0 min	90 min
L. acidophilus				
521	8.5	8.3	8.4	8.1
4796	8.5	9.0	9.0	8.9
4962	8.2	9.0	8.9	8.6
11975	8.1	8.5	8.1	8.3
4356	8.0	8.8	8.8	8.7
33200	9.1	9.2	9.3	9.2
43121	9.4	9.4	9.1	9.1
B. bifidum				
11863	8.8	9.0	8.8	8.9
15696	8.0	8.1	8.1	8.1
35914	8.4	8.7	8.6	8.1

TABLE A2. Individual aminopeptidase activity of selected parents, acid-tolerant mutants, and acid-/bile-tolerant mutants.

substrate[a]	Strain					
	ATCC 33200	LSC 13	LSC13-1 GD4	ATCC 43121	LSC 2	LSC2-1 GD4
Lys	43	35	NA	61	55	33
Arg	34	27	8	54	45	24
Leu	14	8	2	37	33	11
Ala	9	7	1	15	13	5
Met	4	2	1	14	12	6
Val	4	3	NA	3	6	2
Phe	3	3	1	5	4	3
Gly	1	NA[a]	1	2	2	3
Pro	1	1	NA	1	1	NA

[a]activity unit=Δb*/cfu/ml
[a]NA=no detectable activity

TABLE A3. Cell-wall fatty acid composition of parent ATCC 43121 and its isolate LSC2-1 GD4.

	Strain (fatty acid content, %)	
Fatty acid	ATCC 43121	LSC2-1 GD4
10:0	2.07	1.78
12:0	2.08	1.77
14:1	0.87	0.00
14:0	3.33	3.10
16:1	7.34	7.27
16:0	6.62	6.41
18:2	1.96	1.74
18:1	48.49	49.96
18:0	2.96	2.67
19 CYC 9,10/:1	11.90	12.99

TABLE A4. Cell-wall fatty acid composition of parent ATCC 33200 and its isolate LSC13-1 GD4.

	Strain (fatty acid content, %)	
Fatty acid	ATCC 33200	LSC13-1 GD4
10:0	0.90	0.86
12:0	1.62	1.50
14:0	3.14	2.92
16:1	7.23	6.86
16:0	7.33	7.43
18:2	1.84	1.76
18:1	46.51	44.64
18:0	3.10	3.76
19 CYC 9,10/:1	16.54	18.31

TABLE A5. Strains used in cholesterol assay and mean of cholesterol concentration before (reference value) and after (experimental value) 12 hours incubation.

Strain	Reference value (mg/ml)	Experimental value (mg/ml)
ATCC 43121	0.207	0.126
2-1 GD4	0.284	0.128
7-1 GD4	0.273	0.214
7-1 TD5	0.293	0.218
ATCC 33200	0.238	0.163
14-2 TD5	0.247	0.223
13-1 GD4	0.242	0.212

Appendix B. Figures

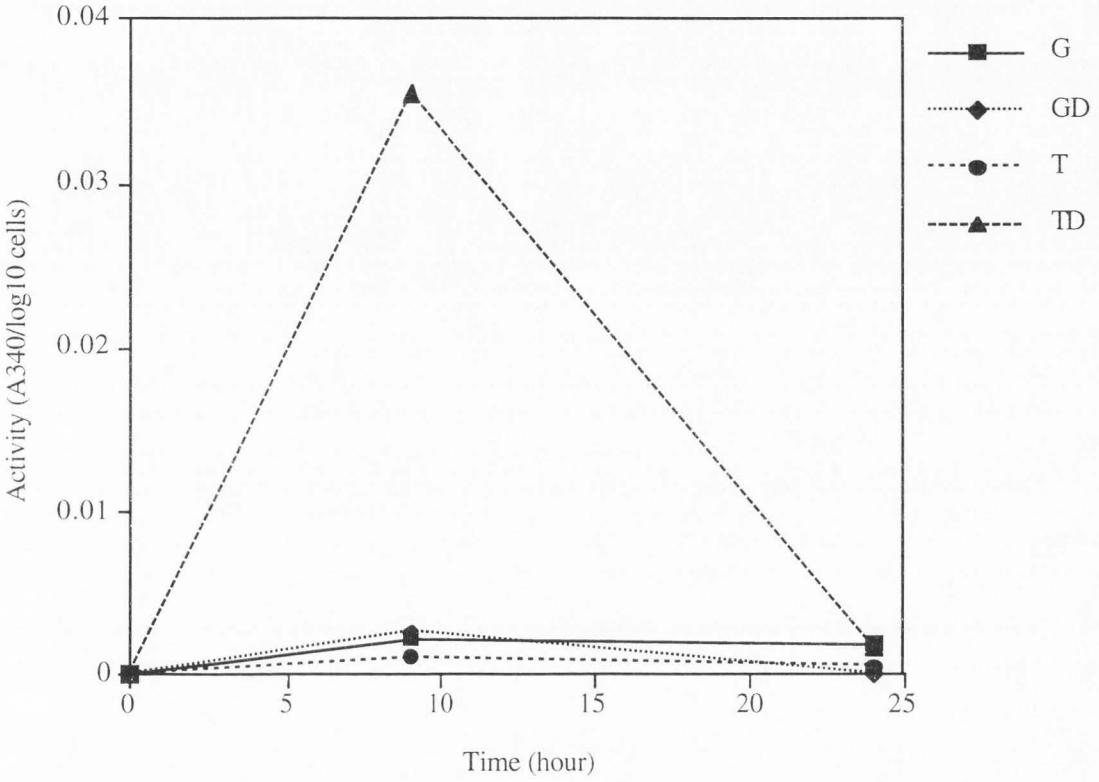

Figure B1. Bile salt hydrolase activity during bacteria growth (ATCC 33200 in MRS+T)

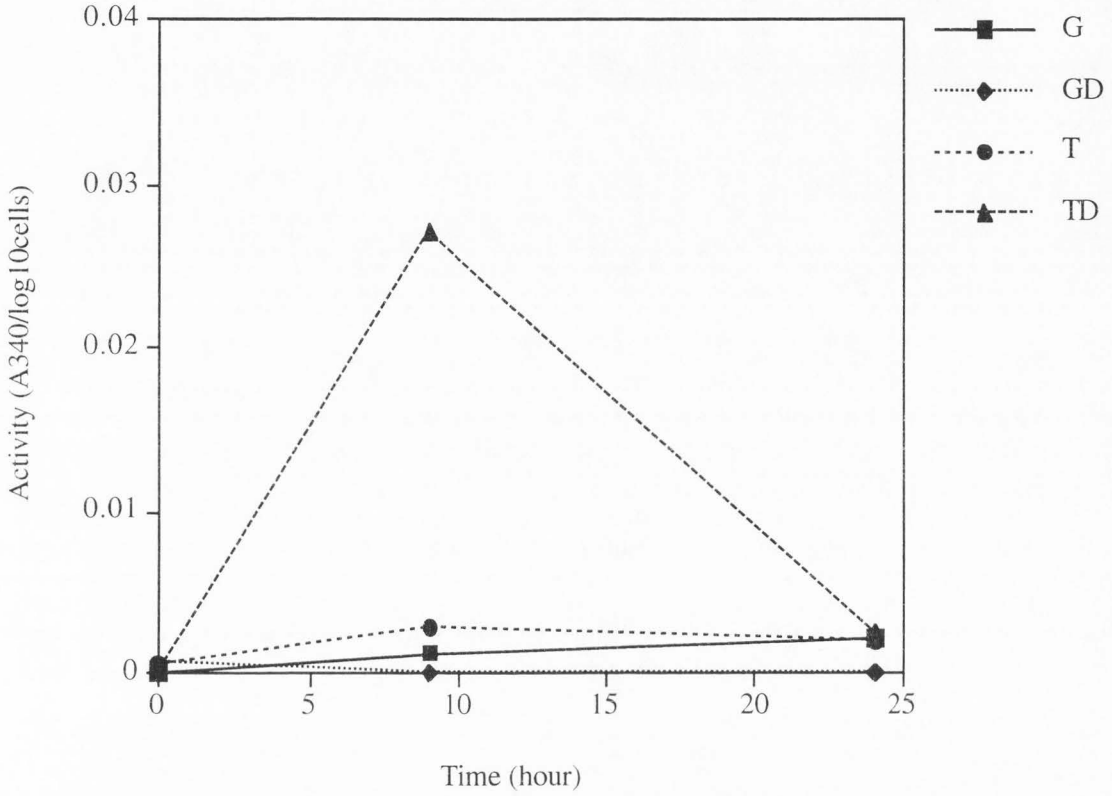

Figure B2. Bile salt hydrolase activity during bacteria growth (LSC13-1 GD4 in MRS+TD)